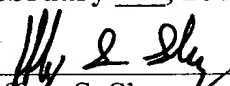




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Docket No.: 28053/38258
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:)	I hereby certify that this paper is being
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Patricia L. CONWAY et al.)	as First Class Mail in an envelope,
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Serial No.: 09/889,085)	Stop Amendment, Commissioner for
)	Patents, P.O. Box 1450, Alexandria,
Filed: January 9, 2002)	VA 22313-1450 on this date:
)	
For: IMPROVED MICROBIAL)	February 22, 2005
PREPARATIONS)	
)	Jeffrey S. Sharp
Group Art Unit: 1651)	Registration No. 31,879
)	Attorney for Applicants
Examiner: Vera Afremova)	

DECLARATION OF IAN L. BROWN
PURSUANT TO 37 CFR § 1.132

I, Ian L. Brown, a resident of the United States, residing at 24 Hancock Court, Basking Ridge, New Jersey 07920, hereby declare that:

1. I am a co-inventor of the invention disclosed and claimed in the above-identified patent application, U.S. Patent Application Serial No. 09/889,085 ("the patent application"), filed January 9, 2002. As such, I am thoroughly familiar with the patent application as originally filed and amended.
2. My qualifications and technical experience are set out in my *curriculum vitae*, a copy of which is attached as Appendix A.
3. I have read and understand the official action from the U.S. Patent and Trademark Office (the "Patent Office") dated August 24, 2004 (the "Office Action"), which was issued in connection with U.S. Patent Application Serial No. 09/889,085. I also have reviewed and understand the patents and publications cited by the Examiner in the Office Action. I make this Declaration to provide information known to me that may be relevant to various claim rejections in the Office Action.

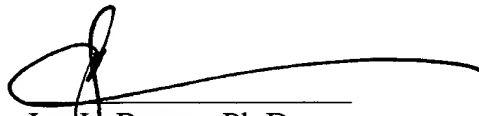
4. The Examiner raised an issue regarding the disclosure of Masuda U.S. Patent No. 5,143,845 in which she suggested that the products of Example 2 comprising microbes incubated in the presence of potato starch would inherently anticipate the applicants' claims. This interpretation is incorrect because Masuda makes clear (and those of skill in the art would recognize) that the potato starch compositions of Masuda are cooked in a manner such that no resistant starch would remain.

5. Specifically, Masuda is directed to a method of preparing microbial cultures on starch media and any of the materials used would be sterilized before their inclusion in the microbial cultures in order to prevent contamination with other cultures. See Example 2, col. 2, lines 57-58 which describes autoclaving the basal media.

6. It is well known that potato starch is resistant in its uncooked state but is digestible (non-resistant) after cooking. Specifically, Rendleman, Biotechnol. Appl. Biochem. 31:171-178 (2000) (Appendix B) describes that uncooked potato starch was only 10.9% degraded to G1 to G7 residues after 8 hours in the presence of alpha-amylase. (Table 2) In contrast, potato starch which had been cooked at 100°C for 30 minutes was 88.6% degraded to G1 to G7 residues. (Table 3) Thus, the cooking process of Masuda destroyed the potato starch granule so that the resultant material is no longer RS2 (uncooked starch). Because the residues referred to are small fragments of seven glucose residues or less (the majority contain two (2) glucose units) they are too small to form into RS3 (retrograded starch). See also, Woodruff and Nicoli Starch gels, Cereal Chemistry 8:243 (1931) (Appendix C) which teaches the gelatinization temperature of potato starch to be only 69-70°C. Finally, Raben et al., J. Clin. Nutr. 60: 544-551 (1994) (Appendix D) states that raw potato starch has a resistant starch content of about 54% while pregelatinized potato starch comprises 0% resistant starch. The pregelatinized potato starch is simply potato starch that has been cooked in water to completely gelatinize the starch and is dried.

7. Because standard autoclave conditions are the use of 121°C at 15 psig for a minimum of 30 minutes (See Effective Use of Autoclaves: Safety Net #26 - UC Davis Environmental Health and Safety - 2-1493) (Appendix E) it is clear that the potato starch components used in the Masuda examples are substantially free of resistant starch.

8. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or document or any patent which may issue thereon.



Ian U. Brown, Ph.D.

Date: 9th February 2005

IAN LEWIS BROWN

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Education: High School Diploma (1976) Forest Park, Ohio, USA.
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Bachelor of Science. (1981) The University of New England, Australia.

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Diploma of Business Studies. (1989) The University of New England.

Master of Science (Biochemistry). (1995) The University of New England.
Thesis entitled "The structure of Australian maize starch"

Doctor of Philosophy (Applied Bioscience, Graduate School of Agriculture).
(1999) The University of Hokkaido, Japan.
Thesis entitled "The development and application of high amylose maize starches
for food, nutritional benefit and public health".

Appointments: 2004 - Visiting Professor, School of Medicine
University of Colorado Health Sciences Center
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2000 - Professorial Fellow, University of Wollongong
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2000 - Member of Advisory Board for Australian Research Council (ARC)
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[University of Wollongong, Australia]

2003 - Senior Director - Nutrition, National Starch and Chemical Company.

Professional Associations: 1985 - 2001 Foundation Member of the Australian Biotechnology Assoc.
1986 - Member of the Royal Australian Chemical Institute.
1986 - Professional Member of the Australian Institute of Food Science & Technology.
1986 - Professional Member of the Institute of Food Technologists
USA.
1990 - Associate Fellow of the Australian Institute of Management.
1994 - Member of the Nutrition Society of Australia.
2000 - Professional Member of the American Association of Cereal Chemists.

Awards: 1975-76 American Field Service Scholarship to study in the USA for 12 months.

1977-80 NSW Department of Education Teaching Scholarship.

1994 RACI [Royal Australian Chemical Institute] **Cereal Chemistry Division Poster Paper Award – Best Visual Presentation.**

1995 RACI [Royal Australian Chemical Institute] **Cereal Chemistry Division Poster Paper Award – Best Poster Contributed by a Commercial Industry Laboratory.**

1995 AIFST [Australian Institute of Food Science & Technology] **Food Industry Innovation Award.**

1999 AIFST [Australian Institute of Food Science & Technology] **The Jack Kefford Award for the Best Paper Published in "Food Australia" in 1998.**

Government:

1998 – 1999. Member of the external technical advisory committee that assisted in the preparation of the Australian & New Zealand Food Authority Full Assessment Report. Proposal P177. Derivation of Energy Factors. February 1999 [Development of Joint Australian New Zealand Food Standards as part of the process of the Review of the Food Standards Code].

2000 - 2001. Member of the Expert Working Group on Dietary Fibre for the Australian & New Zealand Food Authority.

Patents:

Co-inventor of 10 USA patents (more than 32 patents granted internationally in other countries) concerning maize varieties, starch production, resistant starch and dietary fibre utilisation, nutrition, prebiotic functionality of starch and other food ingredients, treatment of human and animal diseases, and the physiological action of starch and other dietary components.

Publications:

Published 27 papers and one book chapter in a variety of international scientific journals on topics concerning cereal science, starch chemistry, food engineering, nutrition and microbiology.

Some 38 other publications have been authored in association with conferences and symposia in Australia and overseas.

Provided educational segments for courses sponsored by the Bread Research Institute, Royal Australian Chemical Institute and University of Wollongong.

Invited & Plenary Presentations:

Australia: Invited to present oral papers at 19 conferences and symposia in Australia.

International: Invited to provide oral papers or chair meetings at 16 international conferences and symposia in Japan, Singapore, Malaysia, New Zealand, USA, Canada, Korea, Ireland and the Philippines.

Committees: Honorary Treasurer for 3rd International Food Hydrocolloids Conference. Sydney. (1996).
Member of the Management Committee of the Cooperative Research Centre for Food Industry Innovation (1996 - 00).

Grants: Successful in obtaining research grants through AJR&DIB and Food into Asia schemes.

Ian Lewis Brown

7 June 2004

Hydrolytic action of α -amylase on high-amylose starch of low molecular mass

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High-amylose starches of low average degree of polymerization (dp 61–71), formed as fine granules by interaction of *Bacillus macerans* cyclodextrin glucanotransferase with α -cyclodextrin (CD) at 2–70 °C, are highly insoluble in water and not gelatinizable under normal cooking conditions (100 °C). Samples of CD-derived starches, both cooked and uncooked, were subjected to hydrolysis *in vitro* by human salivary α -amylase at 37 °C under conditions chosen to resemble those in the human intestinal lumen. Released low-molecular-mass saccharides were determined quantitatively by HPLC and the results compared with those from similar studies with natural starches. Among uncooked starches, CD-derived starch showed very low reactivity towards α -amylase, along with potato starch and a high-amylose hybrid corn starch (64% amylose). Cooking greatly enhanced reactivity of natural starches, but only moderately increased reactivity of CD-derived starches. Susceptibility to hydrolysis of cooked starches increased in the following general order: CD-derived starch (\approx 100% amylose) < 100% corn amylose (isolated by the butan-1-ol method) < hybrid high-amylose corn starch (64–66% amylose) < waxy maize starch (99–100% amylopectin) \sim ordinary corn starch (\approx 25% amylose) < potato starch (\approx 25% amylose).

Introduction

Starch is the most extensively used polysaccharide in food preparations and serves as the principal nutrient in the diet of the Western world. Digestion of starch by humans occurs primarily in the small intestine where two separate families of carbohydrases (enzymes that hydrolyse carbohydrates) are found [1]. One family is introduced into the intestinal lumen and is represented by α -amylases (salivary and pancreatic). The other family is immobilized in the brush-border membrane and includes disaccharidases (such as maltase, lactase and sucrase), trisaccharidases and glucoamylase (amyloglucosidase). Glucoamylase liberates glucose specifically from the non-reducing end of malto-oligo-

saccharides (with greatest affinity for chain lengths of 5–9 glucose units [1]). Pancreatic α -amylase predominates in the small intestine and is located largely in the lumen. α -Amylase specifically catalyses the hydrolysis of α -1,4 glycosidic linkages in starch. Amylose, a linear α -D-glucan, is hydrolysed to maltotriose, maltose and a small amount of free glucose. Hydrolysis of amylopectin, the highly branched form of starch, likewise yields maltotriose, maltose and a small amount of glucose; however, because of the numerous enzyme-resistant branch points, α -limit dextrins (α -1,6-glucosides with 3–6 glucose units) are also formed. Action by α -amylase on maltotriose to produce maltose and glucose is very weak and, therefore, of minor importance in the overall production of maltose and glucose. Maltose appears to be totally resistant to the enzyme. Because only monosaccharides are absorbed through the intestinal wall, maltose must first be hydrolysed to glucose by membrane-bound carbohydrases in the brush border. These same brush-border enzymes contribute to the breakdown of other malto-oligosaccharides. Glucose is absorbed at the rate of 5–20 g/h in the adult duodenum, and at 25–40 g/h in a 45-cm segment of the jejunum.

Although dissolved or gelatinized forms of natural starches react rapidly towards α -amylase, reaction rates of raw or granular forms are much slower and vary according to the source of starch. Leach and Schoch [2] found the following order of increasing resistance of granules towards α -amylase: waxy maize < tapioca < waxy sorghum < sorghum < ordinary corn < wheat < rice < sago < arrowroot < potato < high-amylose corn. At 50 °C, salivary α -amylase hydrolyses granular corn starch at one-eighth of the rate at which it hydrolyses the dissolved form [3]. However, there have been no published studies *in vitro* of enzymic hydrolysis of granular forms of amyloses with a low average degree of polymerization ($\overline{\text{dp}} \approx 55$ –75) under conditions similar to those in the lumen of the human small intestine. Granular forms of this type are readily prepared in good yield from interaction of *Bacillus macerans* cyclodextrin

Key words: α -cyclodextrin, cyclodextrin glucanotransferase, scanning electron microphotography, starch digestion, X-ray diffraction analysis.

Abbreviations used: CD, α -cyclodextrin; CGTase, cyclodextrin glucanotransferase; dp, degree of polymerization; $\overline{\text{dp}}$, average degree of polymerization; SEM, scanning electron microphotograph.

glucanotransferase (CGTase) with α -cyclodextrin (CD) over a wide range of temperatures [4]. Because of the possible usefulness of such amyloses as components of low digestibility in processed foods, a study *in vitro* was initiated in this laboratory to determine the susceptibilities of both cooked and uncooked forms to attack by α -amylase. Conditions of pH, temperature and enzyme concentration were chosen to resemble those conditions in the human small intestine. α -Amylase has optimal activity in near-neutral media (pH 6.9–7.0), similar to the environment in the proximal bowel [5]. Each enzyme molecule requires at least one calcium ion for enzymic activity and to prevent its destruction in the human gut by proteolytic enzymes [6]. Chloride ion is also essential for the action of α -amylases [7], the optimal concentration of Cl^- being $\approx 0.01 \text{ M}$ [3]. When human pancreatic α -amylase is not readily available for use in studies of starch hydrolysis, commercially accessible human salivary α -amylase can be used as an adequate substitute. Various investigators have observed a close similarity between properties of the two enzymes [8,9]. Lee has reported that there is no significant difference in the rate of hydrolysis by salivary, pancreatic and mammary α -amylases [10]. Preduodenal hydrolysis by salivary α -amylase is believed to play only a minor role in the overall digestion of starch in adults. Contribution of salivary α -amylase to enzyme activity in the intestinal lumen is small, partly because of its partial inactivation by gastric acid prior to movement of the enzyme into the duodenum. Nevertheless, in normal adults, as much as 11% of the total α -amylase output in postprandial jejunal fluid is salivary [11].

Experimental

Materials

α -Amylase (EC 3.2.1.1) from human saliva (chromatographically purified; 2500 units/mg of solid, as determined by the Bernfeld method [12]) was obtained from Sigma (St. Louis, MO, U.S.A.). According to the Bernfeld method for determining activity, 1 activity unit of this enzyme liberates the equivalent of 1.0 mg of maltose ($2.9 \times 10^{-3} \text{ mmol}$) from soluble starch in 3 min at pH 6.9 and 20 °C. However, at the higher temperature of 37 °C the effective activity is 1.3 times greater than that at 20 °C. In this laboratory it was determined that 1 activity unit as determined at 20 °C and pH 6.9 will liberate $3.8 \times 10^{-3} \text{ mmol}$ of reducing saccharides [malto-oligosaccharides of degree of polymerization (dp) 2–7 and small amounts of glucose] in 3 min at pH 7.2 and 37 °C. CGTase (EC 2.4.1.19) from *B. macerans* was obtained as an aqueous solution (600 units/ml, pH 7, according to the method of Tilden and Hudson [13]) from Amano International Enzyme Co. (Troy, VA, U.S.A.). CD (8.8% H_2O) was

Table 1 High-amylose starches of low molecular mass formed by the conversion of CD by CGTase

Data for all conversions other than that at 2 °C were taken from [4]. Amylose analysis was by starch-iodine analysis [14]. High-amylose starch prepared at 2 °C was formed by reaction of CD (22.4 g, anhydrous weight) with CGTase (300 units) for 46 days in aqueous solution (215 ml) at pH 7. Streptomycin sulphate (7 mg) was added to prevent microbial growth. A similar yield was obtained in only 3 days when a much larger amount of CGTase (4800 units) was employed. Yield (wt%) refers to the percentage yield calculated on the basis of the anhydrous weight of the starting material.

Reaction temperature (°C)	Yield (wt%)	Moisture content (%)	Amylose analysis	
			dp	Amylose (%)
2	54	11.3	61	104
25	53	13.1	63	99
40	65	14.3	65	96
50	68	13.4	69	98
60	78	13.5	69	98
70	72	9.4	71	94

from Anspec/Ohio (Columbus, OH, U.S.A.). Corn amylopectin (11.5% H_2O) and soluble potato starch were from Sigma; waxy-maize corn starch (10.7% H_2O) and ordinary corn starch (10.1% H_2O) were from Cerestar U.S.A. (Hammond, IN, U.S.A.); potato starch (Avebe; 12.0% H_2O), and Hylon VII (64% amylose; 11.1% H_2O), a high-amylose corn starch, were from National Starch and Chemical Corp., Bridgewater, NJ, U.S.A. Corn amylose ($\approx 100\%$; 6.4% H_2O) was prepared at this Center (the National Center for Agricultural Utilization Research, Peoria, IL, U.S.A.) from corn starch by complexation with butan-1-ol. Low-dp amyloses were prepared by the conversion of CD with CGTase at temperatures ranging from 2 to 70 °C according to methods described earlier [4]. Typical yields and compositions of these amyloses are presented in Table 1. All starches were stored in a chamber maintained at constant relative humidity (31%). Saccharides, used as standards in HPLC analyses, were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Streptomycin sulphate was from Sigma. For buffering purposes, a stock 0.8 M solution of Tris/HCl, pH 7.2, was prepared. Stock solutions of α -amylase (2000 units/ml of 1 mM CaCl_2 solution) were stored in a freezer. Water was distilled and deionized.

Analytical methods

Low-molecular-mass saccharides (dp 1–7) were determined by HPLC on a DuPont Zorbax NH_2 column (4.6 mm \times 250 mm) at 40 °C with acetonitrile/water (13:7, v/v) at 1.0 ml/min and with refractometric detection. The reference standard contained 0.8 mg of each anhydrous carbohydrate/ml of aqueous solution. Calculated yields of saccharides formed by interaction of α -amylase with starch

were based upon the initial amount (in mmol) of anhydro-glucose residues in the substrate.

Low-dp starches were evaluated for both amylose content and dp by spectrophotometric measurement of their amylose-iodine complexes, using the analytical method of Knutson [14] and utilizing the relationships between dp, iodine-binding capacity and wavelength of maximum absorption (λ_{max}) reported by Banks et al. [15]. Amylose contents of natural starches were obtained by similar means. Moisture contents of substrates were determined by heating weighed samples under vacuum at 115 °C for 2 h.

Procedure for enzymic hydrolysis

Into screw-capped culture tubes were placed weighed amounts of starch hydrate and water. A measured volume of stock Tris/HCl buffer (13 μl /ml of reaction mixture) was added to each to impart a Tris concentration of 0.01 M and to ensure that a pH of 6.8–7.0 was maintained throughout enzymic hydrolysis. Cooking, when desired for certain studies, was accomplished at this stage by first immersing the capped tubes in a bath of boiling water (100 °C) for 30 min and then cooling the tubes rapidly to room temperature. After measured volumes of stock α -amylase were added, the tubes were placed in a 37 °C shaker bath for desired periods of time. Reactions were stopped by immersing the tubes in a dry ice/acetone mixture. Reaction mixtures were kept frozen (at or below –30 °C) until time for analysis. No inactivation of α -amylase was necessary. Prior to analysis, each frozen mixture was thawed rapidly and centrifuged briefly at 980 g. An aliquot of the supernatant was diluted to an appropriate volume (usually 10 ml/ml of supernatant), and filtered by syringe through a Millipore HV 0.45- μm filter. A sample of filtered solution was immediately injected into an HPLC equipped for saccharide analysis. The time lapse between centrifugation and HPLC injection was approximately 5 min.

Determination of α -amylase activity at 37 °C

Into a capped culture tube was placed 10.0 ml of a 0.08% (w/v) solution of soluble potato starch containing CaCl_2 (1 mM), Tris/HCl (5 mM), and sufficient NaOH to give a pH of 7.2. After the tube was placed in a 37 °C water bath, a measured amount of α -amylase (0.4 unit of activity based upon a Bernfeld assay at 20 °C) was introduced. At hourly intervals a 1-ml aliquot was removed, cooled quickly to room temperature, filtered by syringe through a 0.45- μm filter, and subjected immediately to HPLC analysis for saccharides produced during enzymic hydrolysis. The time lapse between removal of sample from the bath and its injection into an HPLC column was 4–6 min. Saccharides of dp > 7 were not observed. Graphs of time versus mmol of total saccharides (dp 1–7) and time versus mmol of total

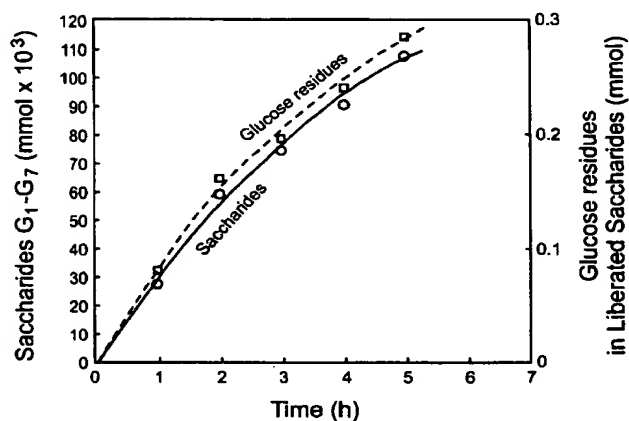


Figure 1 Hydrolysis of soluble potato starch by human salivary α -amylase at 37 °C and pH 7.2

Initial starch solution (10 ml) contained 80 mg of soluble starch (anhydrous basis; \approx 0.493 mmol of glucose residues) and was 1 mM in CaCl_2 and 5 mM in Tris/HCl buffer. Human salivary α -amylase was added to provide an activity of 0.04 unit/ml (based upon a Bernfeld assay at 20 °C [12]). Liberated saccharides (G₁–G₇) were determined by HPLC.

glucose residues in the combined saccharides (Figure 1) permitted a determination of activity not only in terms of total reducing groups produced during the first 3 min of reaction, but also in terms of total glucose residues produced. Liberated glucans appeared to be primarily, if not entirely, straight-chain malto-oligosaccharides, as indicated by their chromatographic retention times. The study revealed that α -amylase activity (in terms of mmol of reducing groups produced) increased by a factor of 1.28 when the temperature was raised from 20 to 37 °C. Thus, at 37 °C and pH 7, a starch solution (10 ml) containing 0.4 unit of α -amylase (based upon a Bernfeld assay at 20 °C) yielded 0.0015 mmol of reducing groups in 3 min. Had the reaction been conducted at 20 °C, only 0.00118 mmol of reducing groups would have been produced. At 37 °C during the first 3 min of reaction, 0.00415 mmol of glucose residues was liberated in the form of saccharides of dp 1–7.

Results and discussion

Studies *in vitro* of starch hydrolysis, designed to provide information about starch digestion in humans, necessitated the use of conditions of temperature, pH, reaction time and α -amylase activity associated with digestion in the intestinal lumen. An assumption was made that the average time for passage of food through the small intestine is 8 h. By means of calculations based upon published data, an α -amylase activity of 13 units/ml of reaction mixture was chosen to represent a typical postprandial activity for intestinal contents. In pancreatic secretion, α -amylase activity at 37 °C has been reported to be about 240 units/ml [9]; and the rate of

Table 2 Reaction of salivary α -amylase at 37 °C and pH 7.0 with uncooked starches

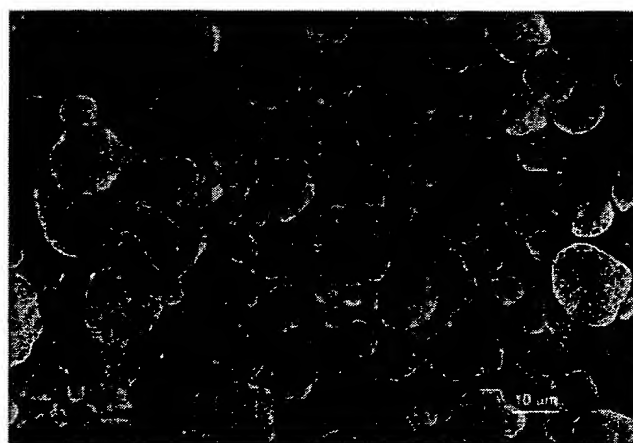
Conditions: α -amylase, 13 units/reaction; volume of mixture, 1 ml; starch, 40 mg (anhydrous weight; equivalent to 0.2467 mmol of glucose residues); Tris/HCl buffer, 0.01 M; CaCl_2 1 mM. Abbreviations: G_1 , D-glucose; G_2 – G_4 , straight-chain malto-oligosaccharides of dp 2–4; G_5 – G_7 , formed only in reactions where the substrate contained an appreciable amount of amylopectin, were probably branched glucans of dp 5, 6 and 7, respectively. Their HPLC elution times were significantly greater than the corresponding elution times of maltopentaose, maltohexaose and maltoheptaose. All starches but corn amylose were granular.

Reaction no/starch	Time (h)	Saccharide yields (mmol of G ₁ residues × 10 ³)								Degradation of starch to G ₁ –G ₇ (%)
		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇	Combined	
Natural starches										
1. Corn, ordinary (≈ 25% amylose)	2	4.9	35.4	21.3	0.5	0.6	1.5	2.2	66.4	26.9
2. Corn, ordinary (≈ 25% amylose)	8	11.5	74.2	28.5	0	1.8	3.8	3.4	123.2	49.9
3. Corn, ordinary (≈ 25% amylose)	20	16.7	114.8	23.4	0	1.7	3.7	3.3	163.6	66.3
4. Waxy maize (99–100% amylopectin)	8	10.3	88.0	42.6	0	1.7	8.4	3.5	154.5	62.6
5. Hylon VII (hybrid corn; 64% amylose)	8	3.4	19.0	3.0	0	0	0	0	25.4	10.3
6. Amylose, 100% (non-granular; from corn)	8	6.7	62.6	17.7	0	0	0	0	87.0	35.3
7. Potato	8	7.7	15.2	2.0	0	0	2.0	0	26.9	10.9
8. Potato	20	8.9	24.1	0.9	Trace	0.5	1.0	0.5	35.9	14.6
Amyloses, CD-derived, low- \bar{dp}										
9. 2 °C product	8	2.8	13.1	1.5	Trace?	0	0	0	17.4	7.1
10. 25 °C product	8	4.5	14.4	2.3	0	0	0	0	21.2	8.6
11. 50 °C product	8	3.9	11.6	2.1	0	0	0	0	17.6	7.1
12. 50 °C product	26	6.8	13.8	0	0	0	0	0	20.6	8.4
13. 70 °C product	8	6.4	23.2	5.4	0	0	0	0	35.0	13.5

flow of this secretion into the duodenum is as high as 1200 ml/day, or 50 ml/h [7]. In the present study, an assumption was made that an average meal is consumed in 1 h and that the ultimate volume of ingested food and drink (including 50 ml of pancreatic juice) is 900 ml. Thus, an upper level of α -amylase activity in the luminal contents would be (240 units/ml \times 50 ml of secretion)/900 ml of luminal contents, or 13 units/ml. In several experiments, time and enzyme concentration were varied to provide information about the influence of such variations on the extent of hydrolysis. Salivary α -amylase was employed as a substitute for pancreatic α -amylase in all of the studies.

Hydrolysis of uncooked starches

All of the starches used in these studies were granular, with the exception of 100% corn amylose which, because of the method employed in its isolation from ordinary corn starch, was amorphous in appearance under a scanning electron microscope. Table 2 presents data on uncooked natural (native) starches (reactions 1–8) as well as on uncooked low-dp amyloses (reactions 9–13) prepared synthetically in this laboratory and described in Table 1. Among the granular natural starches, only Hylon VII and potato starch possessed low susceptibility towards attack by α -amylase. The moderately high susceptibility of 100% corn amylose was probably related, at least in part, to its non-granular structure. X-ray diffraction analysis of this amylose revealed a V pattern, a type normally associated with amylose complexes in which guest molecules are bound within the tunnel-like cavities of starch-chain helices. The complexant butan-1-ol, which had been used in the isolation of amylose

Figure 2 SEM of ordinary corn-starch granules ($\times 470$)

from corn starch, was not present in the amylose, since it had been removed by azeotropic distillation at the conclusion of the isolation process. Examination of the amylose by solid NMR did not reveal the presence of any complexant. Yet, the V pattern suggested strongly that although the conditions of azeotropic distillation were sufficient for removal of complexant, they were not sufficient to allow extensive formation of 'crystalline' amylose, a spherulitic form of starch known to possess a B-type structure and to be highly resistant towards α -amylase [16]. An X-ray diffraction pattern of type B was obtained for Hylon VII, whose granules have smooth surfaces that offer considerable resistance to enzymic attack (Table 2; reaction 5). Low-dp amylose granules derived from CD at 2–60 °C also have

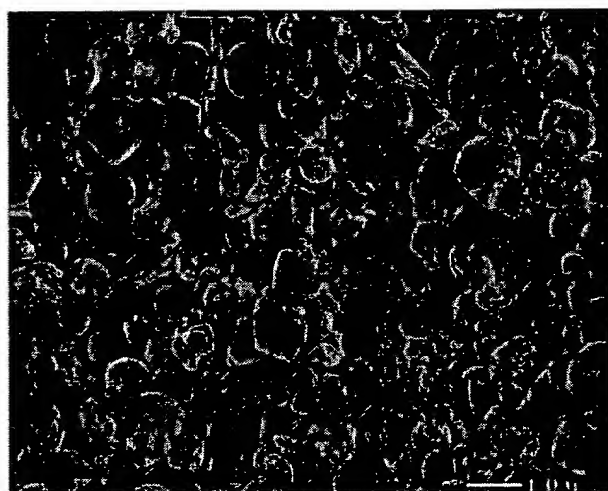


Figure 3 SEM of ordinary corn-starch granules after treatment in aqueous solution with salivary α -amylase at 37 °C ($\times 500$)

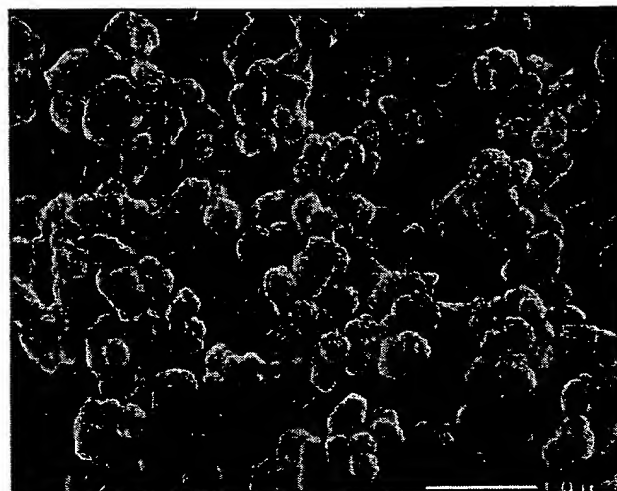


Figure 5 SEM of CD-derived amylose granules prepared at 25 °C ($\times 1000$)

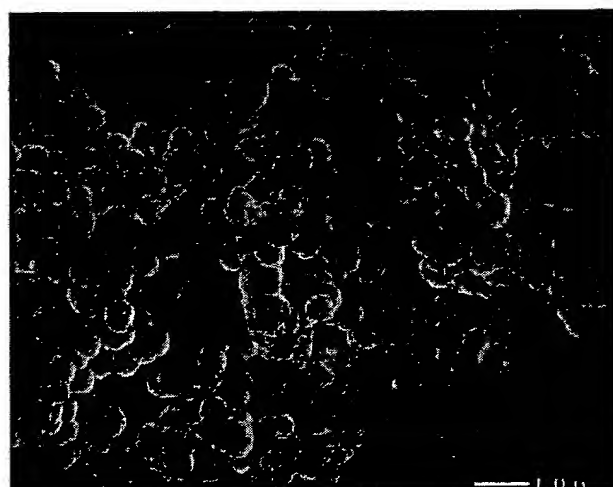


Figure 4 SEM of CD-derived amylose granules prepared at 2 °C ($\times 500$)



Figure 6 SEM of CD-derived amylose granules prepared at 25 °C ($\times 5000$)

type-B diffraction patterns. Their solubility in water is low and their resistance to α -amylase is similar to that of Hylon VII granules and potato-starch granules.

For the natural starches listed in Table 2, the order of increasing resistance to α -amylase is the same as that found by Leach and Schoch [2]. The positions of low- \bar{dp} amyloses in this series are generally slightly higher than that of potato starch: waxy maize < ordinary corn < corn amylose (isolated from corn starch) < potato \sim Hylon VII < CD-derived amyloses. The relatively high reactivity of waxy maize and ordinary corn starch was expected because of the results of earlier studies by Fannon and co-workers [17,18], who investigated surface pores that occur on granules of corn, sorghum and millet starches, but not on granules

of potato, rice, wheat, oat, tapioca, arrowroot and canna. Those investigators proposed that the pores, which appear to be openings that lead to serpentine channels that penetrate into the granule interior, allow access of hydrolysing enzymes and thereby affect the pattern of enzymic attack. Figure 2 is a scanning electron microphotograph (SEM) of corn-starch granules, several of which have surface pores. Figure 3 is an SEM illustrating the extreme pitting that results from α -amylase attack on corn-starch granules. Such pitting has not been observed with granules of CD-derived amyloses. Figures 4, 5 and 6 are SEMs of typical uncooked CD-derived granules. Their shapes are globular and their surfaces are rough, apparently the result of a binding

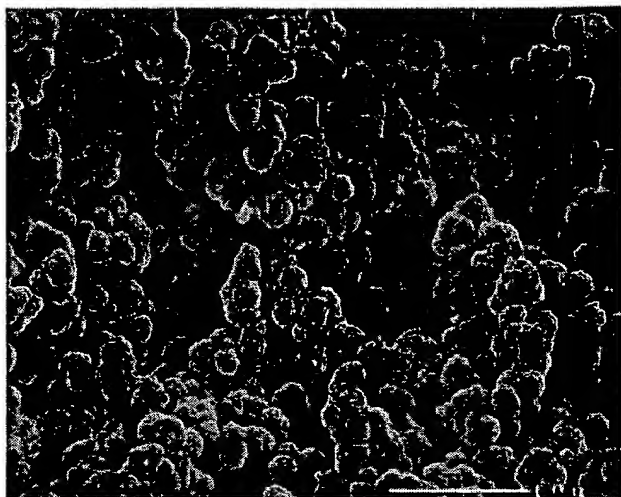


Figure 7 SEM of uncooked CD-derived amylose granules (prepared at 50 °C) that have been treated in aqueous solution with salivary α -amylase at 37 °C ($\times 1250$)

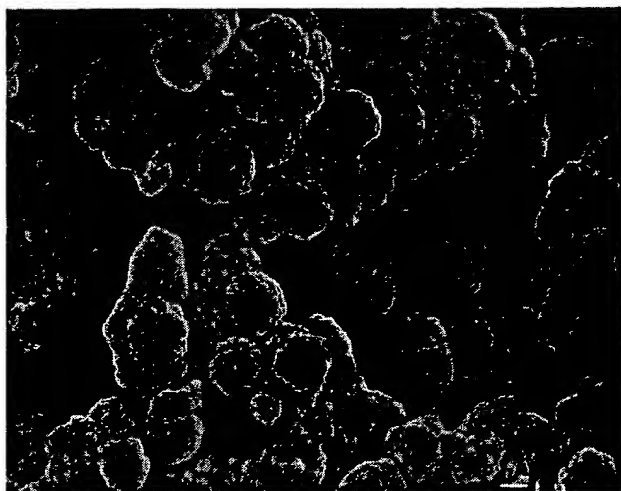


Figure 8 SEM of uncooked CD-derived amylose granules (prepared at 50 °C) that have been treated in aqueous solution with salivary α -amylase at 37 °C ($\times 2500$)

together of numerous very small agglomerates to form much larger agglomerates, or granules. Figures 7 and 8 are SEMs of uncooked CD-derived amylose (prepared at 50 °C) that has been treated with α -amylase. The enzyme caused little change in the spherical character of the granules.

Granules of CD-derived amyloses prepared at low temperature (2–25 °C) were, in general, totally birefringent under a polarizing microscope. However, birefringence occurred only sporadically among granules prepared at 50–70 °C, which would suggest that high preparative temperatures might be detrimental to orderly arrangements

of starch molecules in granular structures. It is not known at this time whether any relationship exists between granular birefringence and susceptibility of granules to enzymic hydrolysis.

Hydrolysis of cooked starches

Table 3 presents results of reactions of salivary α -amylase at 37 °C with starches that have been cooked in aqueous media for 30 min at 100 °C. During cooking, granular starches from ordinary corn, waxy maize or potato become swollen and undergo extensive disruption with concomitant liberation of starch molecules. This process, commonly called gelatinization, greatly lowers the resistance of starch to enzymic attack. Even when cooking merely causes swelling, rather than disruption, of starch granules, as with hybrid corn starches of relatively high amylose content (e.g. Hylon VII and Amylomaize VII), there could be significant diffusion of starch molecules from the swollen granules into the surrounding medium. The magnitude of this diffusion might be large enough to have caused the moderately high levels of enzymic interaction reported in Table 3 (reactions 4, 5, 6 and 7), although not so high as the levels observed for cooked starches from ordinary corn, waxy maize and potato (Table 3; reactions 1, 2, 3, 10 and 11). Consideration should also be given to the possibility that cooking alters the structure of granules from Hylon VII and Amylomaize VII and thereby promotes their penetration by α -amylase.

Cooked 100% corn amylose (Table 3; reaction 8) was hydrolysed by α -amylase to approximately the same extent (34.7%) in 8 h as was the uncooked form (35.3%) reported in Table 2. Increasing both reaction time and enzyme concentration had almost no effect on extent of hydrolysis (Table 3; reaction 9). Cooking apparently had little effect, if any, on the structure of the starch.

Low-dp, CD-derived amyloses did not gelatinize when cooked in water at 100 °C. Dissolution in water at that temperature was only slight, being greatest with amylose prepared at 2 °C. Most of what dissolved at 100 °C precipitated in granular form very slowly over a period of hours when the mixtures were allowed to stand at room temperature. Reactions with α -amylase at 37 °C were initiated immediately after cooking operations to minimize the lessening effect that precipitation of amylose could have on the extent of hydrolysis. All of the cooked CD-derived amyloses (Table 3, see reactions 12–17) were hydrolysed much less effectively than cooked granules of natural starches (Table 3, see reactions 1–7, 10 and 11). Those amyloses prepared at 50 and 70 °C were the most resistant, averaging 22.6% degradation after an 8-h reaction in which the α -amylase activity was 13 units/ml of reaction mixture. In comparison, cooked starches of ordinary corn, waxy corn, Hylon VII, Amylomaize VII and potato underwent 81.8,

Table 3 Reaction of salivary α -amylase at 37 °C and pH 7.0 with cooked starches

Cooking was conducted at 100 °C for 30 min. Conditions: volume of mixture, 1 ml; starch, 40 mg (anhydrous weight; equivalent to 0.2467 mmol of glucose residues); Tris/HCl buffer, 0.01 M; CaCl_2 , 1 mM. Abbreviations: G_1 , D-glucose; G_2 – G_4 , straight-chain malto-oligosaccharides of dp 2–4; G_5 – G_7 , formed only in reactions where the substrate contained an appreciable amount of amylopectin, were probably branched glucans of dp 5, 6 and 7, respectively. Their HPLC elution times were significantly greater than the corresponding elution times of maltopentaose, maltohexaose and maltoheptaose. In reaction 1, a component of molecular mass greater than that of G_7 , probably G_8 (dp 8), was indicated by HPLC analysis. Its contribution to overall yield of saccharides was estimated to be 2.8 mmol of glucose residues, which would raise the level of starch degradation to 77.8%.

Reaction no/starch	α -Amylase (units)	Time (h)	Saccharide yields (mmol of G _i residues $\times 10^3$)								Degradation of starch to G ₁ –G ₇ (%)
			G ₁	G ₂	G ₃	G ₄	G ₅	G ₆	G ₇	Combined	
Natural starches											
1. Corn, ordinary ($\approx 25\%$ amylose)	13	2	9.7	108.3	62.4	0	0.8	3.1	5.0	189.3	76.7
2. Corn, ordinary ($\approx 25\%$ amylose)	13	8	14.2	124.8	51.0	0	1.5	5.4	4.9	201.8	81.8
3. Waxy maize (99–100% amylopectin)	13	8	8.6	119.4	48.9	0	4.5	13.8	0	195.2	79.1
4. Hylon VII (hybrid corn; 64% amylose)	13	8	6.3	95.8	36.6	0	0	0	0	138.7	56.2
5. Hylon VII (hybrid corn; 64% amylose)	13	20	17.7	122.3	14.7	0	0.8	1.6	0	157.1	63.7
6. Hylon VII (hybrid corn; 64% amylose)	51	26	21.6	134.3	1.0	0	0	0	0	156.9	63.6
7. Amylomaize VII (hybrid corn; 66% amylose)	13	8	16.3	102.4	30.3	Trace	0.9	1.5	0	151.4	61.4
8. Amylose, 100% (non-granular; from corn)	13	8	6.1	60.6	18.9	0	0	0	0	85.6	34.7
9. Amylose, 100% (non-granular; from corn)	51	26	13.8	78.0	0.9	0	0	0	0	92.7	37.6
10. Potato	13	8	10.8	141.6	50.7	0	2.2	7.8	5.5	218.6	88.6
11. Potato	13	20	17.9	157.2	25.7	0	2.0	7.0	4.7	214.5	86.9
Amyloses, CD-derived, low-dp											
12. 2 °C product	13	8	4.8	49.0	13.8	0.6	0	0	0	68.2	27.6
13. 25 °C product	13	8	5.1	64.2	21.0	0	0	0	0	90.3	36.6
14. 40 °C product	13	8	7.2	57.8	14.4	0	0	0	0	79.4	32.2
15. 50 °C product	13	8	4.7	39.6	8.1	0.7	0	0	0	53.1	21.5
16. 50 °C product	13	20	7.3	36.2	1.8	Trace?	0	0	0	46.1	18.7
17. 70 °C product	13	8	4.5	44.4	9.6	0	0	0	0	58.5	23.7

79.1, 56.2, 61.4 and 88.6% degradation, respectively, under the same conditions. Among the CD-derived amyloses, observed variations in resistance to α -amylase were possibly related, at least partly, to differences in granule solubility. Those amyloses with the higher solubilities would be expected to exhibit the greater susceptibilities to hydrolysis. Studies now in progress indicate an inverse relationship between average chain length (\bar{dp}) and solubility at 100 °C. This relationship might explain the higher resistance observed for amyloses prepared at 50–70 °C. Figure 9 is an SEM of a CD-derived amylose (prepared at 70 °C) that was cooked and subsequently treated with salivary α -amylase. The treatment had no observable effect on granular shape and very little effect on granular size.

The levels of hydrolysis reported in Table 3 for cooked CD-derived amyloses represent maximum levels. Treatment of the cooked amyloses with α -amylase at 37 °C was made before any significant retrogradation of dissolved amylose could occur subsequent to the temperature reduction from 100 to 37 °C. Any retrograded amylose would probably exhibit an amylolytic susceptibility as low as that of uncooked amylose. Granules of CD-derived amylose are possibly very similar in composition and physicochemical properties to so-called 'resistant starch', produced when ordinary starch gels or amylose gels are given prolonged treatment with α -amylase or mixtures of α -amylase and pullulanase. 'Resistant

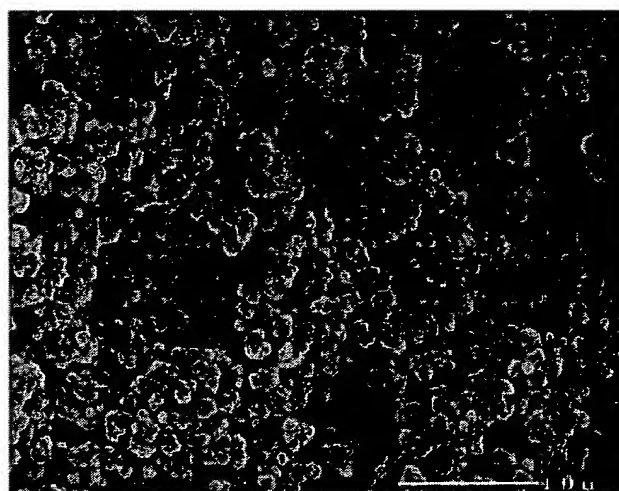


Figure 9 SEM of cooked CD-derived amylose granules (prepared at 70 °C) that have been treated in aqueous solution with salivary α -amylase at 37 °C ($\times 1250$)

starch', prepared in this manner, is in the form of spherulites composed of highly organized linear glucans that possess a B-type X-ray diffraction pattern, a low solubility in water, and, frequently, a low \bar{dp} (≈ 20 –60) [16,19].

Acknowledgments

I am indebted to C. A. Knutson, Jr. for spectrophotometric determinations of amylose content and $\bar{d}p$ of starches; to F. L. Baker for scanning electron microphotography; and to G. D. Grose for X-ray diffraction analyses. Product names are necessary to report factually on available data; however, the U.S. Department of Agriculture (USDA) neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. All programs and services of the USDA are offered on a non-discriminatory basis without regard to race, colour, national origin, religion, sex, age, marital status or handicap.

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pastes had to be heated before gelation was possible, contribute something to the present information on gelatinization temperatures of starches. The subject of gelatinization has occupied many investigators, the details of whose measurements have already been reviewed several times. (Reichert, 1913; Alsberg and Rask, 1924.)

Methods used have consisted of noting microscopic changes in the appearance of the granules, of observing temperatures at which translucency is altered, and of studying changes in viscosity of the paste. Gelatinization temperature has, in this study, been noted in connection with the ability of the cooled paste to form a gel. Our results are in agreement with the statement by Alsberg (1926) that the gelatinization change is continuous over a range of temperature. We have found maximum gelation to occur only after the paste has been heated to a temperature of 90° or above.

The syneresis of gels of varying starch concentration has been measured by Chapman and Buchanan (1930) in corn, wheat, rice and potato starch gels. They state that neither the rate nor the length of the period of heating the suspension affects rigidity or the amount of syneresis in the resulting gels. Hence all their pastes were heated quickly to boiling over a flame and poured out at once. It is not possible to say whether our gels, prepared somewhat differently, were similar in properties to the ones with which Chapman and Buchanan worked, because they confine their measurements to the amount of syneresis and offer no description of the gels' appearance.

Method of Study

Because starch thickening agents are used in the household in only fairly pure form, no attempt was made to purify the samples for these experiments. The cornstarch was a well-known brand of culinary type and the wheat, rice, potato, arrowroot and cassava starches were purchased through a laboratory supply house. In order that results might be interpreted correctly, analyses were made for moisture, ash and nitrogen, the results of which will be found in Table I. No significant amount of nitrogen was found in

TABLE I
ANALYSIS OF THE STARCHES USED

	Moisture %	Ash %	Nitrogen %
Corn	11.30	0.20	0.032
Wheat	10.25	0.13	0.026
Rice	10.80	0.70	0.071
Potato	14.29	0.35	0.049
Arrowroot	13.59	0.28	0.029
Cassava	10.63	0.15	0.012

ion was possible, contribute to our knowledge of gelatinization. Gelatinization has occupied many measurements have already been made (13; Alsberg and Rask, 1924.) Noting microscopic changes in gels, serving temperatures at which changes in viscosity of the starch, in this study, been noted in starch paste to form a gel. Our experiment by Alsberg (1926) that starch gels over a range of temperatures occur only after the temperature is of 90° or above.

Starch concentration has been studied (1930) in corn, wheat, rice and other cereals. It was found that neither the rate nor the temperature affects rigidity or the strength of gels. Hence all their pastes were made in a flame and poured out at once. The gels, prepared somewhat differently from the ones with which Chaplin (1930) they confine their measurements and offer no description of the

Study

Starches are used in the household in many forms. It was made to purify the samples of starch. It was a well-known brand of starch, potato, arrowroot and cassava. It was a laboratory supply house. In this study, analyses were made of the results of which will be given. The amount of nitrogen was found in

Starches Used

Starch	Nitrogen %
Arrowroot	0.032
Cassava	0.026
Potato	0.071
Wheat	0.049
Yam	0.029
Zizania	0.012

any starch; the moisture was about what would be expected in products marketed in paper packages. Ash content indicated that the starches were not pure, but they were so nearly so that differences in gel characteristics could be attributed to the starch component of the only fairly pure material. These observations will be repeated and extended later with purified instead of commercial starches.

One hundred grams of a 5% by weight suspension of starch in water was heated in a 150 cc. test tube which was immersed to a depth below the level of the suspension in a mechanically stirred water bath. The bath consisted of distilled water in a beaker behind which a strong light was placed. The light shining through it made changes in appearance of the contents of the test tube easily discernible. The deep, slender column of starch suspension insured uniform penetration of heat. By means of a glass rod bent to a paddle the starch was stirred occasionally by hand until the stage had been passed where granules were apt to settle to the bottom. Thermometers in the bath and in the tube indicated that there was a uniform difference in temperature between the two of 5° C. throughout the heating period until the bath reached 100°, after which 22 min. more were required to bring the temperature of the starch paste to 99.5° C. the maximum obtainable inside the test tube. At this rate of heating, the starch paste reached a temperature of 90° in about 38 min. The time and temperature intervals are given in the graph (Figure 1).

The tube of starch was removed from the bath after the desired interval of heating and the slight loss by evaporation was adjusted by the addition of water. The paste was viscous but fluid while hot; it was poured into small porcelain crucibles holding 10 g. of paste. Or, in some cases, only a part of the contents of the test tube were poured out and the heating of the remainder was continued for a time after which molds were filled again. At the end of 24 hours the gels were turned out of the molds onto watch crystals and examined for general appearance and firmness. One intact mold of each lot was reserved for photographing. The gels formed in less time than 24 hours but it was convenient to examine all samples one day after they were made.

In certain series sucrose was added to the cold starch suspension and the whole heated together. When 10, 30 and 60 g. of sucrose were added to the 100 g. of starch paste, the corresponding samples were said to contain 10, 30 and 60% of sucrose. It was desired that the ratio of starch to water be kept constant whether or not sucrose was present.

Discussion

Heating temperature required for gelation.—In the course of the gradual heating of the tube of starch suspension there occurred a continuous but slight diminution in opacity. There was visible, however, a quite sudden increase in translucency at a temperature which was specific for each starch. The temperature at which this change occurred was not a sharp one but ranged over 1 or 2 de-

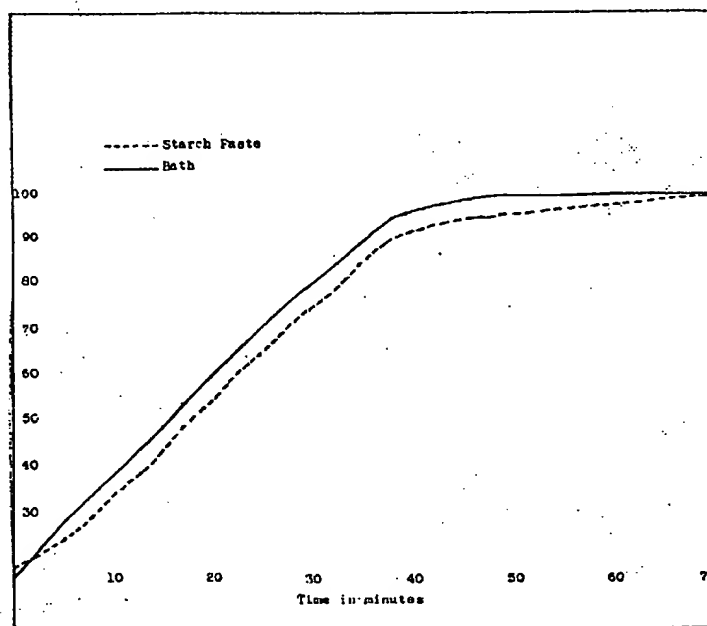


Fig. 1. Rate of Rise in Temperature of Starch Paste and Bath

grees. The paste seemed to have reached its maximum translucency at this temperature; heating either for a longer time or to a higher temperature made but slight difference in its color. This temperature corresponds to what some have called "gelatinization temperature" and for each kind of starch in 5% concentration¹ was:

Corn	86 to 87°C.	Potato	69 to 70°C.
Wheat	87 to 88°	Arrowroot	79 to 80°
Rice	84 to 85°	Cassava	74 to 75°

The change in translucency at what is called here the gelatinization temperature was accompanied by decided swelling of the

¹ It is likely that the temperature of gelatinization as evidenced by changes in translucency varies with the concentration of starch.

tion.—In the course of
ension there occurred
y. There was visible,
ncy at a temperature
perature at which this
aged over 1 or 2 de-

starch granule as shown by microscopic examination, and there was also apparently a change in viscosity under these conditions, though no measurements on the latter are being reported at this time. This temperature was insufficient, however, to permit maximum swelling of the granules, without which seemingly a gel will not form. Samples were poured into molds at the temperatures given above for each starch; the cooled pastes showed evidence of having thickened somewhat at this temperature (bottom row, Plate I) but the gel was weak, granular and showed much syneresis. No evidence of gelation appeared in pastes which had been heated to temperatures 1° or 2° below those given.

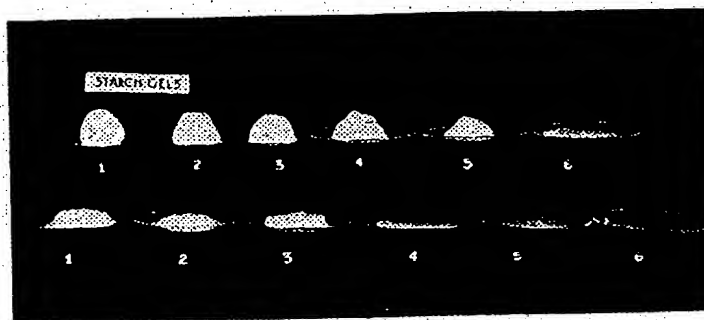


Plate I. Gels of Six Kinds of Starch

1. Corn; 2. Wheat; 3. Rice; 4. Potato; 5. Arrowroot; 6. Cassava.
Above: All pastes had been heated to 99.5° C.
Below: Pastes had been heated to 87°, 88°, 85°, 70°, 80° and 75°, respectively.
(Visible changes in translucency of the pastes occurred at these temperatures.)

Continuing the heating to 99.5° C. gave the three very well-formed cereal starch gels of the top row in Plate I and the soft or quite fluid gels of the root starches also pictured in the top row. Of the three cereal starch gels, the rice was most translucent and most tender as it was cut; cornstarch gave the firmest gel and was chalky white in color even though its hot paste had been a translucent blue. Wheat starch gave results intermediate in value. The potato starch paste was ropy and almost water-clear; the cold gel was quite transparent, too gummy to leave the mold well and in all a poorly formed gel. Arrowroot gave a still more transparent and softer gel. The cassava starch paste was only a viscous fluid even when heated to 99.5°.

If the maximum strength of a gel is desired in a starch, its paste must be heated beyond the temperature at which its translucency suddenly increases. This result suggested that perhaps long continued heating at the gelatinization temperature might

bring about maximum gelation. Each starch was therefore heated in turn for 15 and 30 minutes at its gelatinization temperature; this improved gelation somewhat but did not yield a gel which even approached in firmness the one which had been heated to 90° and removed immediately. Heating the paste to 90°, 95° and 99.5° gave gels indistinguishably alike. This manner of finding the conditions for maximum gel strength was used with each starch in turn with the same general result. Only the wheat starch is so represented in the plates. (Plate II.)

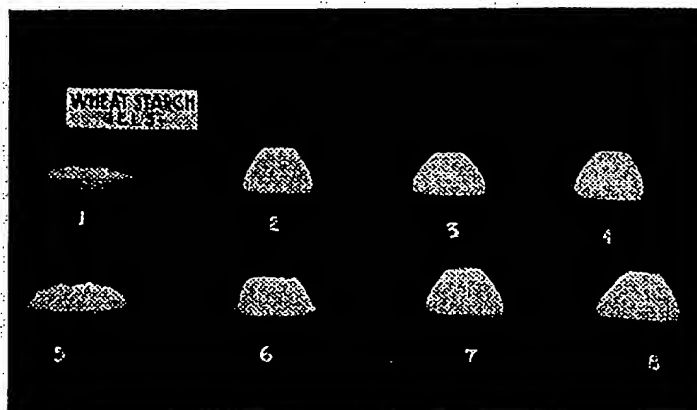


Plate II. Effect upon Gel Formation of Time and Temperature of Heating

- | | |
|---------------------------------|--|
| 1. Paste was heated to 88° C. | 5. Heating continued at 88° C. for 15 min. |
| 2. Paste was heated to 90° C. | 6. Heating continued at 88° C. for 30 min. |
| 3. Paste was heated to 95° C. | 7. Heating continued at 99.5° C. for 15 min. |
| 4. Paste was heated to 99.5° C. | 8. Heating continued at 99.5° C. for 30 min. |

Most starch mixtures probably reach a temperature of 95° to 100° in the course of ordinary cooking. Forty or more records on the inner temperature of baked potatoes have given 100° to 104° as the one for "doneness"; at an inner temperature of 97° the potato seemed still underdone. Biscuits, muffins and butter cake reached a temperature of 100°, yeast bread 98° and even angel food cake, containing as it does large amounts of easily over-coagulable egg white, has been found to have attained an inner temperature of 97°, 98°, 99° and 100° in four different bakings.² Other factors than the gelation of the starch of course contribute to "doneness" in the above products but there is little likelihood that incomplete gel formation occurs commonly, since it has just been shown that a temperature lying between 87° and 90° is sufficient for complete

² Unpublished results by Woodruff.

starch was therefore heated at gelatinization temperature; this did not yield a gel which even had been heated to 90° and then to 90°, 95° and 99.5° gave no sign of finding the conditions with each starch in turn with that starch is so represented

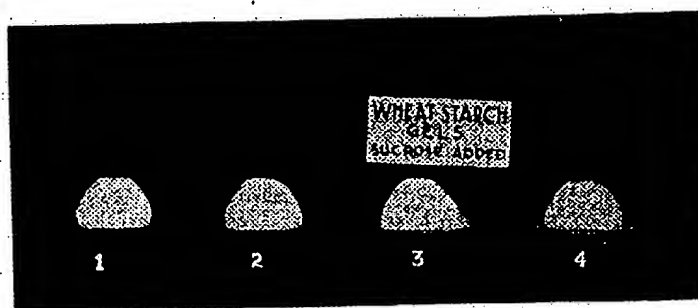


Plate III. Effect of Increasing Sucrose Concentration upon Gel Formation

- | | |
|-----------------------|-----------------------|
| 1. Sucrose none | 3. Sucrose 30 percent |
| 2. Sucrose 10 percent | 4. Sucrose 50 percent |

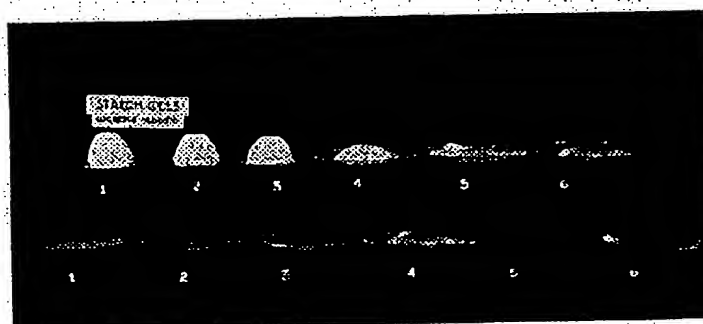


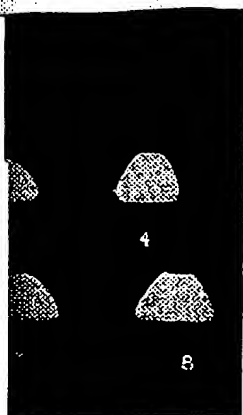
Plate IV. Effect of Sucrose upon Gels of Six Starches

1. Corn; 2. Wheat; 3. Rice; 4. Potato; 5. Arrowroot; 6. Cassava
 Above: Gels containing 30 per cent sucrose, heated to 99.5° C.
 Below: Gels containing 60 per cent sucrose, heated to 99.5° C.



Plate V. Effect of Reducing the Starch Concentration per Unit of Volume by Adding Sucrose and by Adding Water

- Left: Contains 5 grams of wheat starch and 95 grams of water.
 Center: Contains 5 grams of wheat starch and 182 grams of water.
 Right: Contains 5 grams of wheat starch, 95 grams of water and 60 grams of sucrose.
 The volumes of center and right were the same.



Temperature of Heating

- Heated at 88° C. for 15 min.
 Heated at 88° C. for 30 min.
 Heated at 99.5° C. for 15 min.
 Heated at 99.5° C. for 30 min.

temperature of 95° to 100° or more records on have given 100° to 104° temperature of 97° the and butter cake and even angel food easily over-coagulable in temperature of Other factors than is "doneness" in the that incomplete gel been shown that a sufficient for complete

gelation of wheat and corn starches. In rice the temperature lies between 85° and 90° and in potato between 70° and 80°. Arrow-root and cassava formed very poor gels which were little improved by heating to a higher temperature.

Effect of sucrose upon gels.—The failure of starch-thickened fillings to gel in the presence of large amounts of sugar is not infrequently noted. In studying the conditions under which such an effect is produced, additions of different quantities of sucrose were made to pastes, all of which were heated to 99.5° and the resulting gels photographed. Gels containing successively 10, 30 and 50% sucrose showed increasing transparency and tenderness but were well-formed in the three cereal starches. When 60% sugar was added, however, a viscous, syrupy mass resulted but no gel. Plate III, illustrating wheat starch, shows that there is very little outward effect upon the starch of amounts of sucrose up to 50%. Plate IV pictures the rigidity of gel structures of the six starches in the presence of each 30 and 60% sucrose. The three root starches had formed very weak gels even in the absence of sugar; when so little as 10% sucrose was added to their pastes a noticeable softening occurred and 30% flattened out the gel considerably. A syrup was the result when 60% sugar was used with the root starches, as was also the case in the cereal starch pastes.

The volume of a starch-sugar paste containing 5 g. of starch, 95 g. of water and 60 g. of sucrose was 132 cc., whereas the volume of the same amounts of starch and water was approximately 100 cc. The increase in volume had reduced the starch from 5 to 3.8% on the basis of volume. Such a reduction in the ratio of starch to volume would account for some diminution in gel strength but not for the progressive change in color and translucency which was noted as more and more sugar was added. There is evidence in the series of wheat starch gels in Plate V that the sucrose did have a specific effect upon gelation. Photographed there is the syrupy fluid of the paste containing 60% sucrose and a tender but well-formed gel obtained when a starch-water paste was diluted with water to 132 cc., the volume of the sugar-containing one. For purposes of comparison, a gel containing 5 g. of wheat starch and 95 g. of water is also shown in Plate V.

The mechanism of this action by sucrose can only be speculated upon in the light of the present findings. It is possible that the presence of large amounts of sucrose prevents the starch granules from imbibing the water needed for their swelling. The amounts of sucrose employed here are none of them beyond the limits of

In rice the temperature lies between 70° and 80°. Arrows which were little improved

failure of starch-thickened mounts of sugar is not infrequently under which such an amount of sucrose were added to 99.5° and the resulting successively 10, 30 and 50% cy and tenderness but were less. When 60% sugar was added resulted but no gel. Plate that there is very little outflow of sucrose up to 50%. Plates of the six starches in the The three root starches had absence of sugar; when so little pastes a noticeable softening and considerably. A syrup was made with the root starches, as was done.

te containing 5 g. of starch, 132 cc., whereas the volume of water was approximately 100 cc. The starch from 5 to 3.8% on in the ratio of starch to volume in gel strength but not for translucency which was noted here is evidence in the series of sucrose did have a specific effect. Here is the syrupy fluid of the tender but well-formed gel as diluted with water to 132 cc. one. For purposes of comparison starch and 95 g. of water is

sucrose can only be speculated on. It is possible that the presence of sucrose prevents the starch granules from swelling. The amounts of sucrose beyond the limits of

what is used in cooked starch mixtures. The cause of the behavior of sucrose is being studied now by other means.

Summary

When starch-water pastes containing 5% of either corn, wheat, rice, potato, arrowroot or cassava starches were heated only to the temperatures at which the different ones exhibited marked changes in translucency, the cooled pastes did not form gels strong enough to retain the shape of a mold. Maximum gel strength was obtained in each starch at a temperature of 90° or higher. The cereal starch gels were well-formed and had clearly defined outline; root starches gave poorly-formed gels. Photographs constituted a permanent record of the appearance of these gels.

Maximum gelation of starch very probably occurs in baked flour mixtures, in cooked potatoes and in flour-thickened sauces. The final inner temperature of such products has in many cases been observed to be at or near 100° C.

The presence of sucrose in amounts as great as 60% of the weight of the starch paste reduced the mass to a viscous syrup. A starch-water paste of the same volume did not behave as did the starch-sugar-water one. This effect of sucrose is being studied further.

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Resistant starch: the effect on postprandial glycemia, hormonal response, and satiety¹⁻³

Anne Raben, Anna Tagliabue, Niels J Christensen, Joop Madsen, Jens J Holst, and Arne Astrup

ABSTRACT The effect of resistant starch (RS) on postprandial plasma concentrations of glucose, lipids, and hormones, and on subjective satiety and palatability ratings was investigated in 10 healthy, normal-weight, young males. The test meals consisted of 50 g pregelatinized starch (0% RS) (S) or 50 g raw potato starch (54% RS) (R) together with 500 g artificially sweetened syrup. After the R meal postprandial plasma concentrations of glucose, lactate, insulin, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1, and epinephrine were significantly lower compared with after the S meal. Moreover, subjective scores for satiety and fullness were significantly lower after the R meal than after the S meal. Differences in GIP, texture, and palatability may have been involved in these findings. In conclusion, the replacement of digestible starch with RS resulted in significant reductions in postprandial glycemia and insulinemia, and in the subjective sensations of satiety. *Am J Clin Nutr* 1994;60:544-51.

KEY WORDS Potato starch, appetite, palatability, lactate, insulin, gastric inhibitory polypeptide, catecholamines

Introduction

Until recently, starch was believed to be 100% digested in the small intestine—independent of the source, type, and preparation of the starch. However, within the past 10 y it has been found that, despite the fact that pancreatic α -amylase is present in the gut in ample amounts (1), a fraction of the ingested starch passes undigested to the large bowel (2-4). Here the starch undergoes a more or less complete fermentation, resulting in the production and uptake of short-chain fatty acids (acetic acid, butyric acid, and propionic acid) (5, 6). This fraction of the starch has been named resistant starch (RS) and has been defined by the European FLAIR Concerted Action on Resistant Starch (EURESTA) as "the sum of starch and products of starch hydrolysis not absorbed in the small intestine of healthy individuals."

The amount of RS present in starch-rich foods depends on several factors, ie, the source, ripeness, processing, preparation, and storage of the foods. It has been shown that starch from white bread, porridge oats, and cornflakes is almost completely digested in the small intestine (4) whereas native starch from banana and uncooked potato is highly resistant to hydrolysis *in vitro* (7) and *in vivo* (8). When the potato is cooked the starch granules gelatinize and become readily digestible, whereas cooling of the potato reverses this gelatinization process and renders $\approx 12\%$ of the starch resistant to small intestinal digestion (9).

The importance of RS to human health, ie, diabetes, overweight, cardiovascular diseases, or cancer, is still not known. However, because RS—unlike nonresistant starch—is not digested and therefore not absorbed as glucose in the small intestine of healthy humans (8, 9), a reduction in both postprandial glycemia and insulinemia can be expected after the intake of RS compared with digestible starch. Such an effect of RS may be beneficial in the control of diabetes. Moreover, RS may through mechanisms similar to those exerted by soluble dietary fiber influence the amount and rate of absorption of other nutrients in the diet, ie, glucose and fat, which may be beneficial in the control of glycemia or lipidemia.

The potential use of RS as a weight-reducing agent may also be of interest because the energy value of 1 g RS, including the contribution from fermentation products, has been estimated to be only 9.0–9.8 kJ/g, ie, half the value of digestible starch (10). It is therefore tempting to suggest RS as a weight-loss agent; however, the impact of RS on macronutrient balance and appetite control may be less beneficial to weight regulation. This hypothesis is based on the recently developed concepts on the regulation of energy and macronutrient balance. Thus, there seems to be a close regulation between the body's macronutrient stores and the three macronutrients (fat, protein, and carbohydrate) in the diet (11, 12). The maintenance of the body's relatively small carbohydrate stores seems especially crucial to overall energy balance (11). Moreover, recent studies have shown that the control of appetite may be influenced by the amount of available carbohydrate in the diet (13, 14) and that consumption of a certain amount of carbohydrate (or protein) in a meal may be necessary to achieve satiation (15). Increasing the amount of RS in the diet with a resultant decreased amount of absorbed carbohydrate and a decreased carbohydrate-to-fat ratio in the diet may thus lead to

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TABLE 1
Subject characteristics¹

	Denmark (n = 5)	Italy (n = 5)	All (n = 10)
Age (y)	25.6 ± 1.9	21.2 ± 0.7	23.4 ± 1.2
Height (m)	1.80 ± 0.01	1.78 ± 0.02	1.79 ± 0.01
Weight (kg)	71.2 ± 3.0	74.0 ± 3.5	72.6 ± 2.2
BMI ²	22.0 ± 1.1	23.2 ± 0.6	22.6 ± 0.6
Fat mass (%)	18.6 ± 2.4	20.8 ± 1.3	19.7 ± 1.3

¹ $\bar{x} \pm \text{SEM}$.² In kg/m².

a reduction in the satiating power of the diet. This may result in overconsumption and subsequent weight gain. However, if RS has a "dietary-fiber-like" positive effect on satiety (16–18) an increase in the amount of RS in the diet may be beneficial to weight maintenance.

The purpose of the present study was to investigate the acute effects of RS vs digestible nonresistant starch on postprandial changes in glycemia, hormonal response, and subjective sensations of hunger and satiety. The two test starches were given in as pure a form as possible to clarify the role of the starches per se without the interference from other nutrients.

Subjects and methods

The experiment was performed as a joint study within the EURESTA framework. Exactly the same experiment was conducted at two centers: the Research Department of Human Nutrition, The Royal Veterinary and Agricultural University, Denmark, and the Department of Human Nutrition, University of Pavia, Italy.

Subjects

In each center five healthy male subjects (20–31 y of age, normal-weight, nonsmokers, not elite athletes) with no history of obesity or diabetes, participated in the study (Table 1). Females were not included to avoid possible differences due to the menstrual cycle. The subjects' energy needs during the study were determined by using WHO tables according to age, weight, height, and sex. The multiplication factor 1.78 was used to account for medium physical activity level of the subjects (19). The body composition of the subjects was estimated by the bioimpedance method by an Animater (HTS Engineering Inc, Odense) in Denmark and BIA 109 (RJL Systems, Detroit) in Italy. Fat-free mass was calculated by using the equation of Deurenberg et al (20). The study was approved by the Municipal Ethical Committee of Copenhagen and Frederiksberg to be in accordance with the Helsinki-II declaration and all subjects gave written consent after the experimental procedure had been explained to them.

Diets

The two test meals consisted of 50 g raw potato starch (54.1% RS) (R) or 50 g pregelatinized potato starch (100% digestible) (S) mixed into 500 mL diluted artificially sweetened fruit syrup (Table 2). The syrup was based on apple, grape, red current, elderberry, black current, and cherry and commercially purchased from Irma, Denmark. The starches were produced and

TABLE 2
EURESTA reference starch materials¹

Food	Calculated starch values				
	DM	TS	RDS	SDS	RS
	%		% by wt		
Raw potato starch	83.4	81.3	6.0	21.2	54.1
Pregelatinized potato starch	95.1	92.9	87.9	5.2	—

¹ DM, dry matter; TS, total starch; RDS, rapidly digestible starch; SDS, slowly digestible starch; RS, resistant starch. Analyzed from the Dunn Clinical Nutrition Centre. Methods described by Englyst et al (21) and Englyst and Cummings (22).

supplied from l'Institut National de la Recherche Agronomique (INRA), Nantes, France. Both starches appeared as a white flour. Just before consumption the starch was slowly poured into the syrup while mixing it with a hand mixer at the slowest speed to avoid an increase in temperature of the mixture. Several other ways of administration were tested before deciding on these test meals. However, the pregelatinized starch could not be mixed into other liquids without producing a very unpleasant, inedible substance. Because heating would affect the physical properties of the raw starch, a cold-meal preparation had to be used. The starches could thus not be baked into a bread or used in a pasta. The best compromise for these two starches was therefore to mix them with the syrup. For the R meal this resulted in a drinkable mixture whereas the S meal was a porridge-like gel to be eaten by spoon. Although the syrup was sweetened with artificial sweeteners it contained some fruit sugars naturally occurring in the berries and fruits (Table 3).

Each test day was preceded by 3 d on an identical carbohydrate-rich diet (60% of energy as carbohydrate, 28% as fat, and 12% as protein, and 3.5 g dietary fiber/MJ), prepared at the departments from food items according to each subjects' individual energy requirements, and adjusted to the nearest 0.5 MJ. The subjects were instructed to adhere strictly to the diet. If subjects

TABLE 3
Carbohydrate and energy contents in the two test meals
(50 g potato starch + 500 mL diluted fruit syrup)¹

	Test meal	
	R	S
Total starch in 50 g potato starch (g)	40.7	46.5
RS (g)	27.1	0.0
RDS (g)	3.0	44.0
SDS (g)	10.6	2.6
Fruit sugars from syrup (g)	8.4	8.4
Glucose (g)	2.9	2.9
Fructose (g)	4.4	4.4
Sucrose (g)	1.1	1.1
Total digestible carbohydrate (g) ²	22.0	54.9
Total energy (kJ) ¹	367	917

¹ R, raw potato starch; S, pregelatinized potato starch; RS, resistant starch; RDS, readily digestible starch; SDS, slowly digestible starch.

² Not taking fermentation into consideration.

¹ 16.7 kJ/g.

could not consume all the food, they had to bring the leftovers to the departments for weighing and registration. The same food was deducted from the diet during the following preexperimental periods. The subjects were instructed to abstain from strenuous physical activity for the 2 d before the test days. Together with the standard diet this should ensure equally filled glycogen stores and similar macronutrient balance on the 2 test days. The computer databases of foods from the National Food Agency of Denmark (Dankost) and Italy (INN) were used in the calculations of energy and nutrient composition of the test diets.

Experimental protocol

The two test meals were given in a crossover design on separate days with ≥ 1 wk and no more than 6 wk separating the test days. On the test day the subjects arrived at the institute at 0800, with a minimum of physical activity, by car, bus, or train after having fasted for 12 h from the evening before. After subjects voided and were weighed (to the nearest 100 g), bioimpedance was measured. The subjects then rested in the supine position on a bed covered with an antidecubitus mattress with slight elevation of the head. A Venflon catheter (Viggo, Gothenborg, Sweden) was inserted in an antecubital arm vein. After a 10-min rest a fasting blood sample was taken and after a further 20-min rest resting metabolic rate was measured by indirect calorimetry by using a ventilated hood. A second fasting blood sample was taken hereafter and the test meal was then served and consumed within 10 min. Exactly the same time was spent on the two test meals for each individual subject. Postprandial energy expenditure was measured for 5 h (1000–1500 h), and blood samples were taken 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, and 300 min after the meal was consumed. During the postprandial measurements the subjects were allowed to watch light entertainment movies, and to have a break of a maximum of 5 min after 2 and 4 h. During the break the subjects could sit, walk quietly, or go to the toilet. The exact time schedule was noted and repeated on the following test days. Water consumption during the test period was allowed, but the total amount consumed was noted and repeated on the second test day.

Immediately before and every 30 min after the meal, questionnaires to assess hunger, satiety, fullness, and prospective consumption were filled out by each subject. Ratings were made on 100-mm visual analogue scales (VAS) with words anchored at each end, expressing the most positive (ie, good, pleasant) or the most negative ratings (ie, bad, unpleasant) (23). Immediately after the test meals the palatability, taste, aftertaste, texture, and visual appeal of the two test meals were recorded by the subjects using VAS scores. Data on energy expenditure are being published separately (A Tagliabue, A Raben, ML Heijnen, P Deussenberg, E Pasquali, A Astrup, unpublished observations, 1994).

Laboratory analyses

Blood was sampled without stasis through the indwelling antecubital cannula by using iced syringes. Plasma glucose and lactate were analyzed by standard enzymatic methods (24). Plasma glycerol was analyzed after trichloroacetic acid precipitation, essentially as described by Chernick (25). Blood for determination of plasma catecholamines was collected in tubes containing ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and glutathione. Samples were imme-

diately centrifuged for 10 min at $3000 \times g$ and 4°C and the plasma stored at -80°C until determination of catecholamines by a radioenzymatic method (26). Immunoreactive insulin concentrations were measured in plasma with radioimmunoassay kits purchased from Novo, Copenhagen. Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) were determined by radioimmunoassay on plasma extracted with ethanol as previously described (27–29). Samples for the GIP and GLP-1 analyses could not be taken from the Italian subjects. The starch analyses were performed at the Dunn Clinical Nutrition Centre (United Kingdom) by using the methods described by Englyst et al (21) and Englyst and Cummings (22).

Statistical analyses

All results are given as means \pm SEM. Data are presented as changes from basal fasting concentrations by using the second fasting blood sample as the basal concentration. Responses to the two test meals were compared by parametric analysis of variance (ANOVA) for repeated, paired measures with time and diet as factors and with subject nested into diet. The key results are presented with *F* or *t* values and degrees of freedom. Areas under the curves (AUCs) for the 5-h measurement periods were calculated separately for each subject as the difference between the integrated area of the response curve and the rectangular area determined by the basal values (= net response). A paired *t* test was used in the comparisons between two means on the same subject. Regression analyses were performed on the differences between the S and R meals (referred to as S–R) for the 5-h AUCs and means for the VAS scores, peak/nadir values, and $\Delta\text{peak}/\Delta\text{nadir}$ values. This was done to account for the data being paired. The level of significance was set at $P < 0.05$. *Statgraphics* software version 4.2 (Graphic Software Systems, Inc, Rockville, MD) and the *Statistical Analysis Package* (SAS Institute, Cary, NC) were used in the statistical calculations.

Results

Glucose and lactate

Fasting plasma glucose concentrations averaged 4.97 ± 0.08 mmol/L before the S meal and 4.91 ± 0.07 mmol/L before the R meal (NS). A significant interaction between diet and time was observed after the test meals with glucose concentrations increasing nine times as much after the S meal as after the R meal (Δpeak concentrations: S, 3.07 ± 0.29 mmol/L after 30 min; R, 0.36 ± 0.13 mmol/L after 15 min) ($F_{(12,216)} = 25.2$, $P < 0.0001$) (Fig 1). The 5-h AUCs averaged 79.53 ± 27.26 mmol·min/L after the S meal and -42.02 ± 9.51 mmol·min/L after the R meal ($t_0 = 4.51$, $P = 0.0015$). The AUCs for the first 2 h also resulted in a negative area for the R meal (-13.37 ± 6.71 mmol·min/L) compared with the S meal (140.00 ± 21.53 mmol·min/L) ($t_0 = 7.11$, $P < 0.0001$).

Plasma lactate concentrations increased after both test meals with a peak after 45 min for the S meal (1.36 ± 0.09 mmol/L), and after 15 min for the R meal (1.18 ± 0.10 mmol/L) (interaction diet-time: $F_{(1,2,216)} = 25.2$, $P < 0.0001$) (Fig 1). The increase from basal was twice as high after the S (0.52 ± 0.06 mmol/L) than after the R meal (0.24 ± 0.06 mmol/L). Five-hour AUCs averaged 6.9 ± 10.0 mmol·min/L after the S meal and -26.3 ± 8.9 mmol·min/L after the R meal ($t_0 = 3.70$, $P = 0.005$).

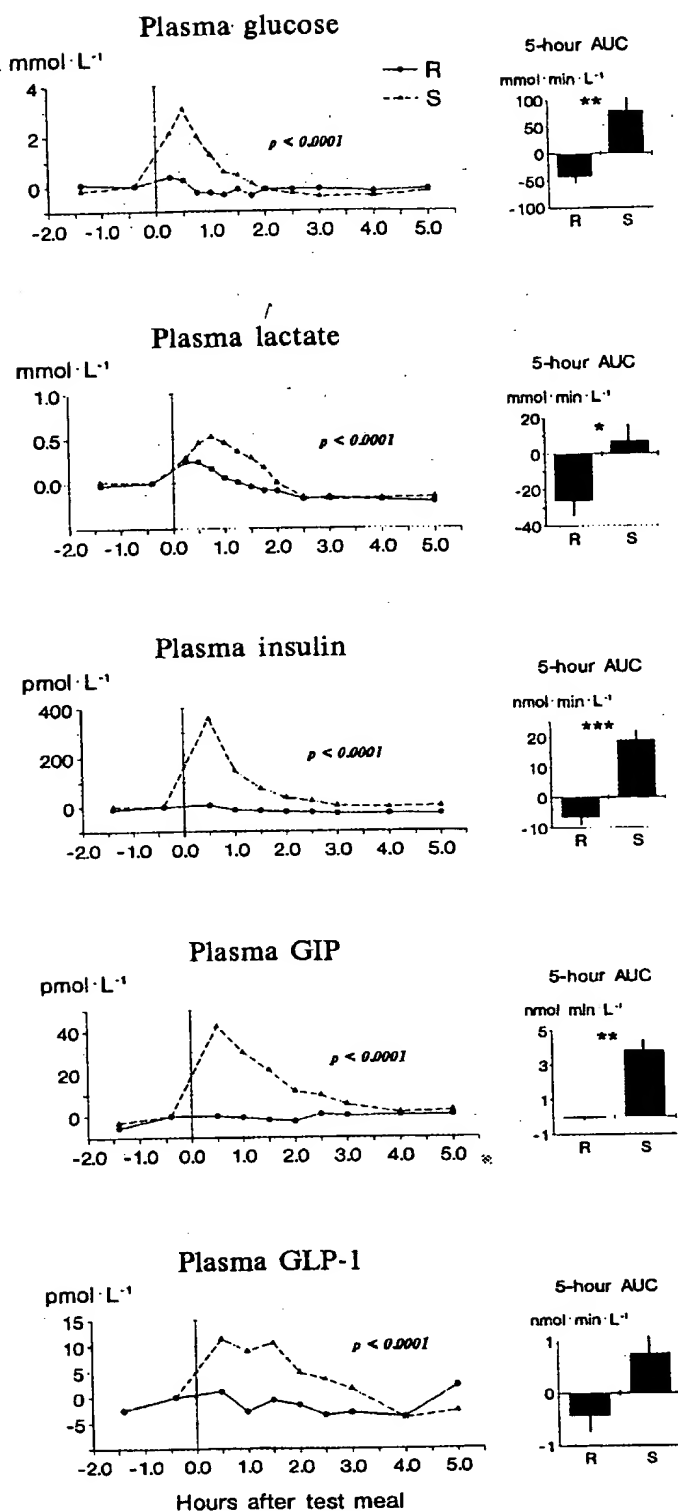


FIG 1. Change in plasma concentrations of glucose, lactate, insulin, gastric inhibitory polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) after a raw potato starch meal [54% resistant starch (RS)] (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (\pm SEM), expressed as differences from fasting concentrations. Left panel (ANOVA): all (time effect: $P < 0.0001$); plasma glucose (diet effect: $F_{(1,18)} = 25.1$, P

Insulin, GIP, and GLP-1

Fasting plasma insulin concentrations averaged 53.7 ± 5.0 pmol/L before the S meal and 75.4 ± 10.0 pmol/L before the R meal (NS). A significantly different response pattern was observed after the two test meals. Compared with fasting concentrations plasma insulin increased by a factor of six to 355.4 ± 68.5 pmol/L after the S meal, but to only 84.6 ± 7.2 pmol/L after the R meal (interaction diet-time: $F_{[8,143]} = 15.14$, $P < 0.0001$) (Fig 1). The AUCs averaged 19.1 ± 4.0 nmol·min/L after the S meal and -6.6 ± 1.2 nmol·min/L after the R meal ($t_0 = 4.90$, $P = 0.0008$). Also, the AUCs for the first 2 h resulted in differences between the S (18.4 ± 2.8 nmol·min/L) and the R meals (-1.4 ± 1.1 nmol·min/L) ($t_0 = 5.34$, $P = 0.0005$).

Plasma GIP showed the same response pattern as plasma insulin (Fig 1). Thus compared with fasting values, GIP increased by a factor of 10 after the S meal whereas no increase was observed after the R meal (interaction diet-time: $F_{[8,63]} = 14.6$, $P < 0.0001$). The AUC S-R for GIP and lactate were positively correlated ($r = 0.91$, $P = 0.03$).

Plasma GLP-1 increased significantly after the S meal whereas no changes were observed after the R meal (interaction diet-time: $F_{[8,57]} = 4.7$, $P = 0.0002$) (Fig 1). The difference in the AUCs after the R and the S meals was nearly significant ($t_0 = 2.56$, $P = 0.06$). The AUC S-R for GLP-1 was negatively correlated with GIP ($r = -0.94$, $P = 0.02$) and lactate ($r = -0.88$, $P = 0.052$) and Δ peak S-R for GLP-1 negatively correlated with GIP ($r = -0.91$, $P = 0.03$).

Triglycerides (TG) and glycerol

Plasma triglyceride concentrations showed a similar response pattern after the two test meals (Fig 2). Thus, TG decreased slightly after both meals with a nadir after 2.5 h and returned to baseline after 5 h (time effect, $F_{[9,135]} = 4.1$, $P < 0.001$). Triglyceride concentrations were significantly correlated with insulin for the S-R AUCs ($r = 0.65$, $P = 0.04$) and the S-R Δ peak/ Δ nadir values ($r = 0.83$, $P = 0.006$).

A tendency to a different response pattern for plasma glycerol was found after the two test meals. Thus plasma glycerol decreased after the S meal to a nadir after 1.5 h whereas no decrease was observed after the R meal (interaction diet-time: $F_{[8,144]} = 1.9$, $P = 0.06$) (Fig 2). After 5 h glycerol concentrations had increased by 59.3 mmol/L for the S meal and by 39.8 mmol/L for the R meal compared with fasting concentrations. The AUCs were not significantly different after the two test meals. The AUC S-R for glycerol was negatively correlated with glucose ($r = -0.75$, $P = 0.01$), whereas peak S-R for glycerol was positively correlated with peak S-R triglyceride ($r = 0.72$, $P = 0.02$).

< 0.0001 ; interaction between diet and time: $F_{[12,216]} = 25.2$, $P < 0.0001$); plasma lactate (diet effect: $F_{[1,18]} = 36.7$, $P < 0.0001$; interaction between diet and time: $F_{[12,216]} = 25.2$, $P < 0.0001$); plasma insulin (diet effect: $F_{[1,18]} = 39.9$, $P < 0.0001$; interaction between diet and time: $F_{[8,143]} = 15.1$, $P < 0.0001$); plasma GIP ($n = 5$) (diet effect: $F_{[1,8]} = 27.3$, $P < 0.0001$; interaction between diet and time: $F_{[8,63]} = 14.6$, $P < 0.0001$); plasma GLP-1 ($n = 5$) (interaction between diet and time: $F_{[8,57]} = 4.7$, $P < 0.0001$). Right panel: areas under the curves (AUC). *** $P < 0.05$, ** $P < 0.01$, * $P < 0.001$.

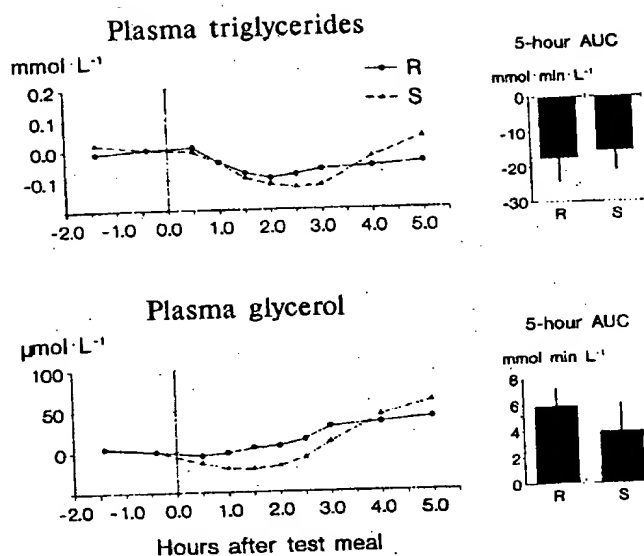


FIG 2. Change in plasma concentrations of triglycerides and glycerol after a raw potato starch meal (54% resistant starch [RS]) (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (\pm SEM) expressed as changes from fasting concentrations. Left panel (ANOVA): all (time effect: $P < 0.001$); plasma glycerol (interaction between diet and time: $F_{[8,144]} = 1.9$, $P = 0.064$). Right panel: areas under the curves (AUC).

Epinephrine and norepinephrine

No significant differences were observed in plasma norepinephrine (NE) after the two test meals (Fig 3). However, a significantly different response pattern for E was found after the two meals. Thus, no changes in plasma E concentrations were observed after the R meal, whereas E increased significantly from 2 to 4 h after the S meal (interaction diet-time: $F_{[9,144]} = 2.68$, $P = 0.0064$) (Fig 3). The differences in AUCs were not significantly different for E or NE.

Satiety scores

Significant differences were found for satiety and fullness (interaction diet-time: $F_{[11,198]} = 1.95$, $P = 0.03$ and $F_{[11,198]} = 2.30$, $P = 0.012$) after the two test meals (Fig 4). Thus the subjects felt more satisfied and more full after the S meal than after the R meal.

Simple regression analysis showed no correlations between any of the mean satiety scores and the blood indexes when 5-h AUCs were used. When peak and nadir values were included, mean hunger S-R was significantly correlated with Δ peak S-R for lactate ($r = 0.68$, $P = 0.03$) whereas peak GIP S-R was correlated with peak fullness ($r = -0.87$, $P = 0.057$) and with nadir prospective food consumption ($r = 0.88$, $P = 0.046$).

Evaluation of the test meal

The subjects found that the S meal looked less appetizing ($t_9 = 3.52$, $P < 0.001$), had a more unpleasant taste ($t_9 = 2.84$, $P < 0.05$), and had a firmer texture ($t_9 = -5.38$, $P = 0.0004$) than the R meal (Table 4). They did not find that the aftertaste or overall palatability were different between the meals. Simple regression analysis on the S-R scores showed correlations be-

tween aftertaste and mean satiety ($r = 0.65$, $P = 0.04$), hunger ($r = -0.64$, $P = 0.049$), and fullness ($r = 0.62$, $P = 0.054$).

Discussion

Marked differences in plasma concentrations of substrates and hormones as well as in palatability and satiety scores were observed after the two test meals. Overall, the S meal greatly stimulated plasma concentrations of glucose, insulin, and gastrointestinal hormones whereas no or only a modest stimulation of these indexes was observed after the R meal.

Plasma glucose increased nine times more after the S meal than after the R meal. This was not entirely unexpected on the basis of the analytical data on digestibility of the two test starches. However, the differences in postprandial glycemia were somewhat larger than would be expected from the clinical analyses. Thus, the ratio between the peak glucose response after the R and S meal was 1:9 (0.36/3.07 mmol/L), whereas the ratio between the amount of readily digestible carbohydrate was 1:5 (11.4/54.4 g) (Table 3). The reason for this difference is not readily apparent but may be connected with differences in the stimulation of gastrointestinal factors and insulin after the two meals.

Despite the minor increase in plasma glucose after the R meal, the increase in plasma lactate concentration amounted to 50% of the increase after the S meal. This may be due to the fructose present in the test meal (4.4 g) or to a part of the glucose being converted to lactate via nonoxidative pathways in the splanchnic region (30).

Also, plasma insulin increased markedly after the S compared with the R meal, producing a ratio between Δ peak values of 30:1 (182/6 pmol/L). This difference in insulin response is far greater

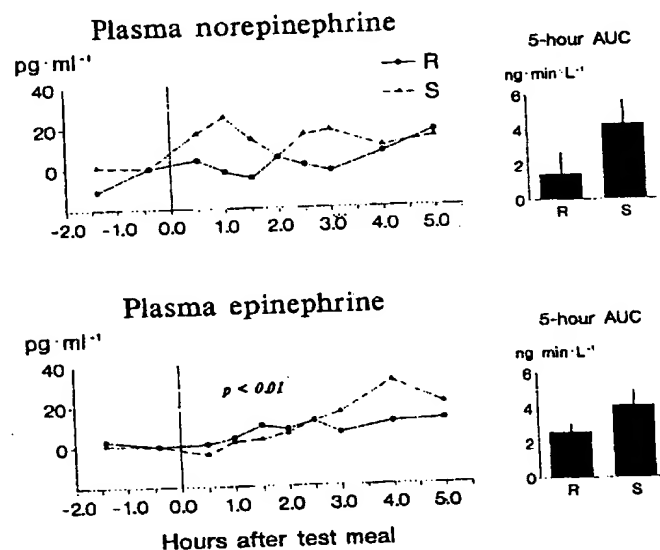


FIG 3. Changes in plasma concentrations of norepinephrine and epinephrine after a raw potato starch meal (54% resistant starch [RS]) (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (\pm SEM), expressed as differences from fasting concentrations. Plasma epinephrine (time effect: $F_{[9,144]} = 8.37$, $P < 0.0001$; interaction between diet and time: $F_{[9,144]} = 2.7$, $P = 0.0064$). Right panel: areas under the curves (AUC).

than would be expected from the difference in glucose load and glucose response after the two meals. In trying to explain this it may be useful to look at the changes in gastrointestinal hormones. Not only did the R meal result in a much lower blood glucose response compared with the S meal, but at the same time there seemed to be no effect on either the proximal (GIP) or distal (GLP-1) incretins in contrast with the increase in these hormones after the S meal. Both GIP and GLP-1 are known to be potent stimulators of insulin secretion (31). The large difference between the meals in these hormones may therefore explain the difference—beyond what can be expected from the glucose-stimulated insulin secretion—in insulin response between the two meals.

The subjective feelings of satiety and fullness were also significantly influenced by the digestibility of the starch. Thus, the pregelatinized and fully digestible potato starch meal resulted in greater feelings of satiety and fullness compared with the resistant and slowly digestible starch meal. Already, 1–1.5 h after the raw potato starch meal the subjective scores were back to fasting concentrations, whereas the satiating power of the S meal lasted 2.5–3 h postprandially.

It has previously been stated that changes in satiety after a carbohydrate load may be mediated through an effect of plasma glucose or hepatic glycogen concentration on specific glucosensitive cells in the brain (32) and studies have demonstrated a satiating effect of carbohydrate per se (14). We found no significant correlations between the changes in plasma glucose and in the four satiety scores. In a recent study from our department in which a high- and a low-fiber meal were given to 10 healthy subjects, the AUCs for glucose and satiety scores were not significantly correlated either (18). In the present study differences in hunger ratings between the two meals were in fact correlated with differences in Δ peak lactate concentration, which may re-

TABLE 4
Subjective evaluation of the test meal¹

	R	S
Visual appeal (0: good, 10: bad)	5.3 ± 0.7	8.1 ± 0.5 ²
Taste (0: pleasant, 10: unpleasant)	6.0 ± 0.8	7.2 ± 0.8 ¹
Aftertaste (0: none, 10: much)	5.4 ± 1.1	7.0 ± 0.7
Texture (0: firm, 10: loose)	7.7 ± 0.8	3.4 ± 0.8 ¹
Palatability (0: good, 10: bad)	7.4 ± 0.6	8.2 ± 0.6

¹ $\bar{x} \pm \text{SEM}$. R, raw potato starch meal; S, pregelatinized potato starch meal.

² ¹Significantly different from R (unpaired *t* test, *df* = 9); ²*P* = 0.007, ³*P* = 0.02, ⁴*P* = 0.0004.

fect the rate of nonoxidative glucose disposal. In the present study carbohydrate was the only energy source in the test meals, but because an increased energy load in a meal has also been shown to result in an increased satiating power of the meal (33), it may be that the differences in satiety sensations were due to a different net energy load in the meals. A firmer conclusion may, however, require more direct measurements of glycogen stores or glucose disposal in order to explain changes in subjective satiety scores.

Gastrointestinal hormones have previously been connected with satiety and obesity (34, 35). In the present study some correlations were found between GIP and satiety scores but not between GLP-1 and satiety. The correlations found indicate that GIP may decrease satiety and increase hunger, which is in contrast with the previously suggested satiating power of GIP. In the present study, however, data on GIP were only available for five subjects. The correlations must therefore be considered with

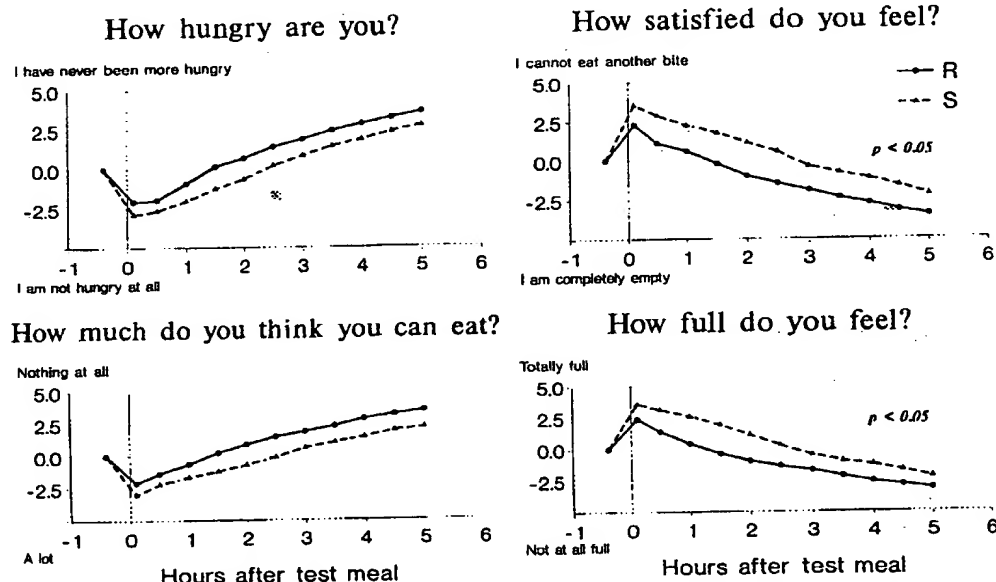


FIG 4. Subjective satiety scores after a raw potato starch meal (54% resistant starch [RS]) (R) and a pregelatinized potato starch meal (0% RS) (S) in 10 healthy, normal-weight, male subjects. All data are mean (\pm SEM), expressed as differences from fasting values. ANOVA: all (time effect: $F_{(11,198)} = 1.95$, $P = 0.03$); satiety (top right); diet effect: $F_{(1,18)} = 4.94$, $P = 0.04$; interaction between diet and time: $F_{(11,198)} = 2.30$, $P = 0.012$).

some caution, and need to be confirmed before any further conclusions can be made.

Because the volume of the test meals and the time spent on consuming the meals on the 2 test days were similar, these factors cannot have influenced the satiating power of the two meals. However, the gelatinization of the S meal and the fact that it was a solid meal may have resulted in a reduction in gastric emptying rate (36) and in increased feelings of satiety and fullness after this meal compared with the liquid R meal. Also, the different ratings of taste, visual appeal, and texture may have influenced satiety and fullness ratings. In fact, positive correlations were found between aftertaste and satiety and fullness. This partly confirms the previous study by Hill et al (23) in which increased hunger was found with increased preference (palatability) of a meal. However, in their study there was no effect on fullness ratings of the meal preference (23).

The effect of RS on satiety shown in the present study may be considered a negative effect of RS with regard to appetite regulation and perhaps also weight control. Replacement of digestible starch with nondigestible starch in the diet may pose a risk of increasing the overall fat-to-carbohydrate ratio in the diet. Because a high-fat, low-carbohydrate diet has been shown to increase energy intake and body weight under ad libitum conditions, whereas a low-fat, high-carbohydrate diet has been shown to decrease spontaneous food intake and result in unexpected weight reductions (37–39), a replacement of digestible starch with RS may be undesirable in long-term weight regulation. Whether RS in a mixed meal has the same effect on appetite as in the present study remains, however, to be elucidated. Moreover, it is not possible to predict the effect on 24-h appetite sensations from the present 5-h results. The RS not digested in the small intestine will at a later stage, ie, 7–12 h after ingestion, be digested and fermented by bacteria in the large intestine, resulting in the production and uptake of short-chain fatty acids (5, 6, 40). Whether this may contribute to increasing satiety remains to be elucidated.

In conclusion, the intake of RS resulted in significantly lower postprandial plasma glucose, lactate, insulin, GIP, and GLP-1 responses and in a reduction of the satiating power compared with digestible starch. The changes in plasma glucose were not correlated with satiety or fullness, but correlations were found between GIP and satiety scores. Differences in texture and palatability of the test meals may also have influenced the subjective satiety ratings. Further studies are needed to clarify the effect of RS in a mixed meal, both acutely and long term. ■

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EFFECTIVE USE OF AUTOCLAVES

SafetyNet #26

Steam sterilization of materials is a dependable procedure for the destruction of all forms of microbial life. They are common laboratory tools that must be properly used to be effective. This establishes guidelines for the effective use of steam sterilizers (autoclaves) for the decontamination of cultures and other potentially biohazardous materials.

Successful Components of Sterilization

This should include validation of decontamination effectiveness. Validation of effectiveness includes monitoring temperature, pressure and cycle duration time for each cycle and providing periodic decontamination challenges (quality assurance), i.e. use of biological indicators. A logbook should be maintained to record autoclave use and be available for inspection.

- **Temperature-** This denotes heating in an autoclave employing saturated steam under a pressure of approximately 15 psi to achieve a chamber temperature of a least 121°C (250°F) for a minimum of 30 minutes.
- **Time-** The time is measured after the temperature of the material being sterilized reaches 121°C (250°F).
- **Contact-** Steam must contact all areas of the load. Autoclave bags should be left partially open during decontamination to allow steam to penetrate into the bag. Air pockets or inadequate steam supply will cause sterilization failure.
- **Containers-** Materials that are to be decontaminated should be carried to the autoclave in leak proof containers. Containers used to hold material while being autoclaved are described below.
 - Primary Containers – Clear autoclave bags come in a wide variety of sizes. They are usually placed in a secondary container during decontamination cycles to catch liquids that may drain out of the bag.
 - Secondary Containers – Plastic or stainless steel containers are commonly used to contain material during autoclaving. Polypropylene plastic pans with 6-12 inch sides are favored over polyethylene and polystyrene because it can withstand autoclaving without melting. Stainless steel containers are durable and a good conductor of heat.
- **Indicators-** These are tools used to validate the decontamination process.
 - Chemical indicators change color after being exposed to 121°C (250°F), but they have no time factor.
 - Tape indicators can only be used to verify that the autoclave has reached normal operating temperatures for decontamination.
 - Biological indicators are designed to demonstrate that an autoclave is capable of killing microorganisms. A load test using *Bacillus stearothermophilus* should be performed monthly.

- **Recordkeeping-** Records of maintenance, logs, calibration results and *Bacillus stearothermophilus* load tests should be kept for a minimum of three years.

Autoclave Training and Operation

Principal investigators, or supervisors must train and qualify their staff for operation of steam autoclaves for decontamination of materials. Qualified autoclave users should understand the time, temperature, pressure relationships required for proper materials decontamination. Additional training on handling materials to be decontaminated should also be provided. Supervisors should maintain a permanent record of training provided to their staff.

- Personnel should wear proper personal protective equipment, i.e. heat resistant gloves, eye protection and a lab coat, particularly when unloading the autoclave.
- Regularly inspect your autoclave components for proper operation. Follow the autoclave manufacturer's recommendations for inspections and service. Autoclaves should be inspected on a regular basis and temperature gauges should be calibrated at least annually. If a problem is found, promptly notify your area supervisor who will call for maintenance. **Do not operate an autoclave until it has been properly repaired.**
- **Never place sealed containers in an autoclave.** Large bottles with narrow necks can simulate sealed containers if filled with too much liquid.
- **Do not** autoclave items containing solvents, volatile or corrosive chemicals (phenol, trichloroacetic acid, ether, chloroform, etc.) or any radioactive materials. Call EH&S (752-1493) if you have questions regarding waste disposal.
- After loading and starting the autoclave, processing time starts after the autoclave reaches normal operating conditions of 121°C (250°F) and 15 psi pressure.
- Decontamination conditions vary with type of load therefore processing times will vary according to the conditions. A minimum of 30 minutes is needed to decontaminate biological waste.
- At the end of a decontamination cycle make sure that the pressure in the autoclave chamber is near zero before opening the door. Slowly crack open the autoclave door and allow the steam to gradually escape from within the autoclave.
- Take care when opening autoclave doors. Opening the autoclave door too quickly may result in glassware breakage and/or steam burns on your skin.
- Allow materials inside the autoclave to cool for 10 minutes before removing them from the autoclave.
- After autoclaving, waste can be disposed of as solid waste.

Wastes that are classified as medical waste (potentially/known infectious to humans and sharps waste) must be treated in accordance with the Medical Waste Management Act. For additional information, contact your EH&S Safety Advisor, EH&S at 752-1493 or ehs@ucdavis.edu.

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